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# ALTERATIONS IN BRAIN 5-HYDROXYTRYPTAMINE METABOLISM DURING THE 'WITHDRAWAL' PHASE AFTER CHRONIC TREATMENT WITH DIAZEPAM AND BROMAZEPAM

R.A. AGARWAL, Y.D. LAPIERRE, R.B. RASTOGI & R.L. SINGHAL

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Ontario K1N 9A9, Canada

1 Daily administration of diazepam or bromazepam (10 mg/kg) for 22 days significantly increased the activity of mid-brain tryptophan hydroxylase by 36% and 39%, respectively. The concentration of tryptophan was also enhanced in the mid-brain region of rats subjected to benzodiazepine treatment.

2 Chronic therapy with either of the two anti-anxiety agents enhanced the endogenous levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in cerebral cortex, hypothalamus, pons-medulla, mid-brain and striatum.

3 Whereas diazepam treatment decreased (13%) the activity of monoamine oxidase in mid-brain, bromazepam failed to exert any effect, suggesting that the observed elevation in 5-hydroxyindoleacetic acid levels is not associated with enhanced deamination of 5-hydroxytryptamine.

4 Discontinuation of treatment for 48 h significantly decreased the activity of mid-brain tryptophan hydroxylase to levels that were significantly lower than those seen for benzodiazepine-treated and normal rats. The concentrations of mid-brain tryptophan and 5-hydroxytryptamine were also reduced in various brain regions examined.

5 Withdrawal from diazepam or bromazepam therapy further augmented the levels of brain 5-hydroxyindoleacetic acid.

6 The results demonstrate that the depressant effects on behaviour of these agents are accompanied by increased metabolism of 5-hydroxytryptamine in the brain. Withdrawal from these minor tranquillizers, on the other hand, reduces the synthesis of this indoleamine.

## Introduction

Considerable attention has recently been focussed on the role of 5-hydroxytryptamine (5-HT) in certain behavioural states (Sheard, 1969; Redmond, Mass., Kling, Graham & Keirmenzian, 1971; Boelkins, 1973). Indeed, evidence supports the notion that changes in this important indoleamine are involved in sleep processes (Jouvet, 1969) and anxiety states (Wise, Berger & Stein, 1970). In addition, brain 5-HT has been implicated in the control of seizures in animals (Jenner, Chadwick, Reynolds & Marsden, 1975). Benzodiazepines represent a group of compounds that are extensively used as sedatives, hypnotics, central muscle relaxants, antiepileptic, pre-anaesthetic and antianxiety agents. However, the beneficial effects of benzodiazepines in anxiety states are temporary and continuous administration of the drugs over a prolonged period may be required. Furthermore, psychophysiological studies have shown that upon cessation of therapy, there is a clinically observable return of previous anxiety levels (Rastogi, Lapierre & Singhal, 1976c; Lapierre, unpublished data). Of greater interest is the finding that in certain cases, the post-drug anxiety was even greater than the pre-treatment level which would seem to be in line with the 'rebound' phenomenon noted by Borland & Nicholson (1975) following discontinuation of nitrazepam treatment.

Despite the profound influence of benzodiazepines on the central nervous system, no unified concept is yet available that could account for various pharmacological effects of these drugs by a specific action on a given neuronal system. Recently, chronic administration of diazepam or bromazepam was found to decrease spontaneous locomotor activity and the turnover of noradrenaline and dopamine. These changes were accompanied by decreased activity of tyrosine hydroxylase and increased levels of the catecholamines in hypothalamus, pons-medulla, mid-brain and striatum (Rastogi, Lapierre & Singhal, 1976b). The levels of striatal homovanillic acid (HVA), the chief metabolite of dopamine, were significantly decreased in rats treated chronically with either diazepam or bromazepam. Catecholaminergic projections in the brain as well as the 5-

hydroxytryptaminergic system are diffuse systems linking together many cerebral structures. Since available evidence indicates involvement of 5-HT in behavioural manifestations of anxiety as well as sleep processes (Jouvet, 1969; Stein, Wise & Belluzi, 1975), the present study was undertaken to examine the effects of chronic treatment with diazepam and bromazepam on the activity of tryptophan hydroxylase (TPH) as well as the levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in certain discrete brain regions. In order to elucidate the neurochemical basis of the 'rebound' phenomenon seen during the withdrawal phase after repeated exposure to benzodiazepines, the influence of 48 h discontinuation of treatment also was studied on the biosynthetic capacity of 5-hydroxytryptaminergic systems.

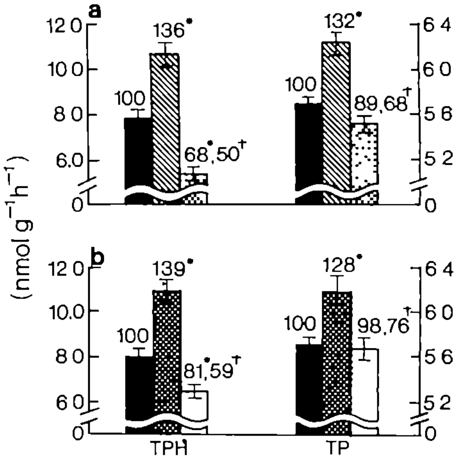

**Figure 1** Effect of (a) chronic diazepam, (b bromazepam and 48 h withdrawal on mid-bra tryptophan hydroxylase (TPH) and tryptophan (TP) Each column represents the mean of 6 rats in the group. Vertical lines show s e means. Animals were injected daily with diazepam or bromazepam (10 mg/kg) subcutaneously for 22 days and killed 6 h after the last injection. Groups of animals treated with either drug for 20 days were subsequently withdrawn for 48 h ('withdrawn' rats). Numbers above columns express results as percentages, taking the values of control rats as 100%. Solid columns = control rats; hatched columns = diazepam-treated rats; stippled columns = diazepam withdrawn rats; cross hatched columns = bromazepam-treated rats; open columns = bromazepam withdrawn rats.

* Statistically significant difference when compared with the values of normal control rats ($P < 0.05$).

† Statistically significant difference when compared with the corresponding values of diazepam- or bromazepam-treated rats ($P < 0.05$)

## Methods

Albino male Sprague-Dawley rats (100–115 g) were housed in a group of 6 per cage and exposed to regular alternate cycles of 12 h light and darkness under constant environmental conditions (24°C, 60% relative humidity). The animals had access to Master Laboratory Chow and water *ad libitum.*

In the first series of experiments, animals were injected subcutaneously (10 mg kg$^{-1}$ day$^{-1}$) with diazepam or bromazepam dissolved in propylene glycol, in a fixed volume of 0.2 ml/100 g for a period of 22 days. Control rats received an equal volume of propylene glycol and animals were killed 6 h after the last injection of the drug or the vehicle. In another series, groups of rats which had been exposed either to diazepam or bromazepam for 20 days were maintained without any treatment for an additional period of 48 h when the animals were killed for biochemical assays.

### *Sample preparation and biochemical assays*

Rats were killed between 09 h 30 min and 12 h 30 min by the 'near-freezing' technique of Takahashi & Aprison (1964). Following decapitation, the brain was dissected into five different regions (cerebral cortex, pons-medulla, hypothalamus, mid-brain and striatum) according to the procedure of Glowinski & Iversen (1966). To determine the activity of TPH and monoamine oxidase (MAO), the mid-brain was homogenized in 20 volumes of 0.28 M sucrose. The assay for TPH was carried out under linear kinetic conditions according to the procedure of Peters, McGeer & McGeer (1968) with minor modifications as described earlier (Rastogi & Singhal, 1974). The concentration of tryptophan was determined in mid-brain according to the procedure of Hess & Udenfriend (1959). The activity of MAO was assayed according to the method of Wurtman & Axelrod (1963) as described in an earlier study (Rastogi, Lapierre & Singhal, 1976a). In order to determine the levels of 5-HT and 5-HIAA, the brain tissue was extracted and assayed according to the procedure of Curzon & Green (1970).

Data were analysed for significance of differences by Student's *t* test.

## Results

### *Effects of chronic benzodiazepine treatment and subsequent withdrawal on mid-brain tryptophan hydroxylase activity and tryptophan levels*

Data shown in Figure 1 demonstrate that repeated exposure of rats to diazepam and bromazepam for 2

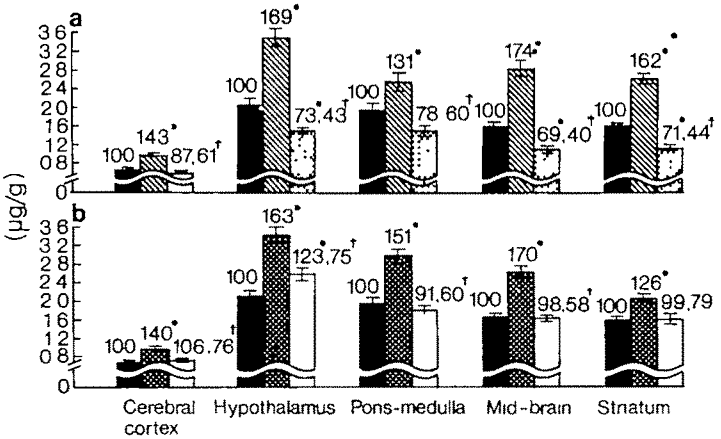

**Figure 2** Effect of (a) chronic diazepam, (b) bromazepam and 48 h withdrawal on 5-hydroxytryptamine concentrations in certain discrete brain regions of rats Each column represents the mean of 6 rats in the group. Vertical lines show s e means. Solid columns = control rats; hatched columns = diazepam-treated rats; stippled columns = diazepam withdrawn rats; cross hatched columns = bromazepam-treated rats, open columns = bromazepam withdrawn rats. For other details see legend to Figure 1.

days significantly elevated the activity of mid-brain TPH to 136% and 139% of the normal values, espectively. Since brain 5-HT synthesis may be ontrolled by the availability of dietary tryptophan, he concentration of this amino acid also was xamined in control as well as in rats subjected to hronic benzodiazepine therapy. As seen in Figure 1, the levels of brain tryptophan were also significantly increased following treatment with either of these anxiolytic drugs. Upon cessation of therapy for 48 h, the activity of TPH was reduced to the values that were significantly lower than those seen for the corresponding 'treated' and normal controls. The tryptophan concentration in mid-brain of rats withdrawn from diazepam and bromazepam treatment also was significantly reduced by 32% and 24% respectively, the values were, however, statistically indistinguishable from those of normal animals.

### *Changes in 5-hydroxytryptamine concentrations of ertain discrete brain regions*

Administration of diazepam or bromazepam for 22 days increased the steady state levels of 5-HT in all egions of the brain examined (Figure 2). In the case of diazepam, the maximal rise was observed in mid-brain, hypothalamus and striatum. Discontinuation of diazepam treatment for 48 h significantly reduced 5-HT concentrations in all regions when compared with the values of the 'treated' group. In hypothalamus, mid-brain and striatum, 5-HT concentrations were significantly lower than those seen for even normal ats. Treatment with bromazepam considerably increased 5-HT concentrations in pons medulla also. Withdrawal from bromazepam lowered the 5-HT concentration to normal limits except in the hypothalamus where it was still significantly higher than the normal values.

### *Effects on 5-hydroxyindoleacetic acid concentrations*

As with 5-HT, the concentration of 5-HIAA was significantly increased in virtually all regions of the brain studied in rats exposed chronically to diazepam or bromazepam (Figure 3). The rise in this brain indoleamine metabolite was more pronounced in animals receiving bromazepam. Withdrawal for 48 h following 20 days of benzodiazepine therapy further augmented the levels of 5-HIAA in several regions of the brain. Again, the magnitude of change was somewhat greater in rats exposed to bromazepam in all regions examined, except striatum.

### *Changes in mid-brain monoamine oxidase activity*

In order to examine whether the observed rise in 5-HIAA was associated with an enhancement in the activity of the deaminating enzyme MAO, the influence of chronic treatment with diazepam or bromazepam as well as subsequent withdrawal was investigated on this metabolizing enzyme in mid-brain. Data in Table 1 show that treatment of rats with diazepam slightly (13%) but significantly reduced the activity of mid-brain MAO. Upon withdrawal from this minor tranquillizer, the level of this enzyme was

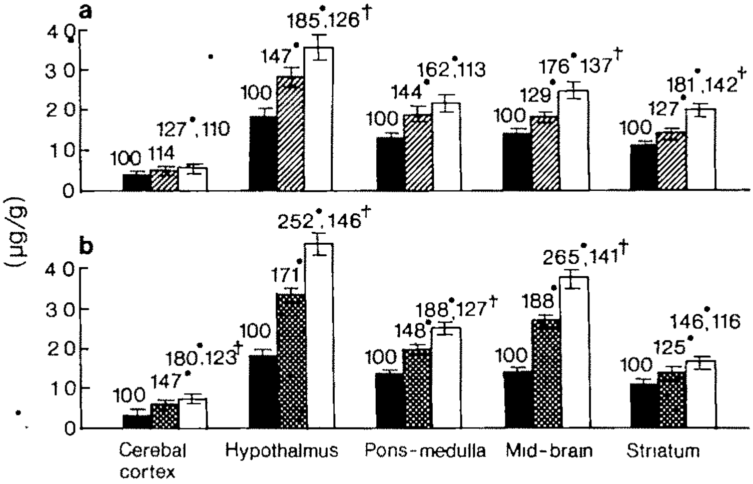

**Figure 3** Influence of (a) chronic diazepam, (b) bromazepam and 48 h withdrawal on 5-hydroxyinoleacetic acid concentrations in certain discrete brain regions of rats. Each column represents the mean of 6 rats in the group. Vertical lines show s.e means Solid columns = control rats; hatched columns = diazepam-treated rats; stippled columns = diazepam withdrawn rats; cross hatched columns = bromazepam-treated rats, open columns = bromazepam withdrawn rats. For other details see legend to Figure 1.

significantly increased to 123% of the values seen for diazepam-treated rats. Data in Table 1 also demonstrate that neither bromazepam nor withdrawal from bromazepam treatment exerted any significant effect on the activity of mid-brain MAO.

**Table 1** Effect of chronic diazepam, bromazepam and 48 h withdrawal on mid-brain monoamine oxidase (MAO)

| *Treatment* | *MAO (nmol $g^{-1}$ $h^{-1}$)* | *% Change* |
|---|---|---|
| Control | 116.72 ± 1.19 | (100) |
| Diazepam | 101.55 ± 2.10 | (87*; 100) |
| Diazepam-withdrawn | 124.89 ± 1.71 | (107; 123†) |
| Bromazepam | 113.96 ± 1.18 | (98; 100) |
| Bromazepam-withdrawn | 117.52 ± 1.02 | (101; 103) |

Each value represents the mean ± s.e. mean of 6 animals in the group. Rats were injected daily with diazepam or bromazepam (10 mg/kg) subcutaneously for 22 days and killed 6 h after the last injection. Groups of animals treated with either drug for 20 days were withdrawn for 48 h ('withdrawn' rats). Data in parentheses express results in percentages taking the values of control rats as 100%.

* Significantly different from normal control values at $P < 0.05$.

† Significantly different from values of diazepam-treated rats at $P < 0.05$.

## Discussion

Several drugs acting on the central nervous system appear to interact specifically with the mechanism that are involved in the maintenance of dynami equilibrium of neurotransmitter substances. Recen studies have shown that acute treatment with diazepam and nitrazepam decreased noradrenaline and dopamine turnover in certain brain regions and antagonized the increased catecholamine turnover induced by electro-foot shock stress (Taylor & Laverty, 1969). Fennessy & Lee (1972) found no change in the endogenous levels of 5-HT and 5-HIAA in brains of mice 30 min after oral administration of diazepam (7 mg/kg). Wise, Berger & Stein (1972) demonstrated that daily treatment with oxazepam (20 mg/kg) for 6 days decreased 5-HT turnover in rat brain as evidenced by elevated levels of [$^{14}$C]-5-HT and [$^{14}$C]-5-HIAA in rats pretreated with [$^{14}$C]-5-HT by the intraventricular route. Furthermore, Lidbrink Corrodi, Fuxe & Olson (1973) found decreased turnover of 5-HT in cortex of rats treated with a single injection (25 mg/kg) of chlordiazepoxide. This is in line with our recent work in which significantly decreased synthesis of 5-HT, as evidenced by lowered tryptophan and TPH activity, was found in mid-brain of rats killed 2 h after a single injection (10 mg/kg) of diazepam (unpublished data). This is not too intriguing since opposite effects on central monoamine turnover have been demonstrated following acute and chronic administration of several psychoactive agents (Javoy Thierry & Kety, 1968; Schildkraut, Winokur

Draskoczy & Hensle, 1971). Thus, any apparent discrepancy between our data and those reported earlier may be explained on the grounds of different doses and duration of treatment. It is probable that acute or subacute administration of oxazepam for 6 days (Wise *et al.*, 1972), by lowering the turnover of brain 5-HT, reduces the concentrations of this amine at the vicinity of corresponding receptor sites; this in turn, by feedback mechanisms may result in induction of TPH enzyme in mid-brain (a region rich in 5-hydroxytryptaminergic cell bodies) as has been documented after repeated administration of a number of psychoactive drugs by Knapp (1975). The increased TPH activity may thus not be in direct response to the effect of chronic diazepam treatment, but may reflect an adaptive change as a consequence of alterations in 5-HT neuronal activity resulting initially from decreased 5-HT turnover after short term treatment with these anxiolytic drugs.

Changes in brain tryptophan levels are relevant to 5-HT mediated functions because in the brain, tryptophan is known to modulate the synthesis of 5-HT (Tagliamonte, Tagliamonte, Perez-Cruet, Stern & Gessa, 1971; Hamon & Glowinski, 1974). Since tryptophan binds avidly to albumin in plasma (McMenamy & Oncley, 1958), the observed rise in brain tryptophan levels might be the result of displacement of tryptophan from the binding site in albumin molecule by benzodiazepines which are known to be bound to plasma protein (80–90%) (Goodman & Gilman, 1975). The elevated levels of endogenous tryptophan as well as the activity of TPH may underlie the enhanced synthesis of 5-HT in brains of rats exposed to diazepam or bromazepam. In addition, there might be several other mechanism(s) through which repeated exposure to benzodiazepines could increase 5-HT turnover e.g. acceleration of 5-HT release, blockade of 5-HT receptors or enhanced nervous activity in 5-hydroxytryptaminergic neurones. However, with the information available in the literature, the latter possibility seems to be favoured. Our data on the elevated brain 5-HT and 5-HIAA concentrations are consistent with the previous reports (Fernstrom, Shabshelowitz & Faller, 1974; Jenner *et al.*, 1975). The increased concentrations of 5-HIAA might be due to increased turnover of 5-HT and impeded egress of this metabolite from the brain. Chase, Katz & Kopin (1970) observed a reduced efflux of labelled [$^{14}$C]-5-HIAA from the brain after its intraventricular injection in rats pretreated with diazepam. The possibility also remains that observed elevation in 5-HIAA levels might be related to increased activity of the deaminating enzyme. However, our data showed that whereas diazepam treatment decreased MAO activity, administration of bromazepam exerted no effect on this metabolizing enzyme.

Behavioural states of excitation with increased locomotor activity have been observed after the administration of drugs that are known to enhance the activity of catecholaminergic neurones in the brain (Ernst, 1967; Carlsson, 1970). Drugs that reduced central catecholamine transmission produced sedation and hypoactivity. In contrast, a state of general excitement with increased locomotor activity was noted after pharmacological and neurological manipulations which selectively lowered brain 5-HT (e.g. inhibition of 5-HT synthesis by *p*-chlorophenylalanine: Sheard, 1969; Brody, 1970) and destroyed 5-hydroxytryptaminergic neurones by electrolytic lesions placed in the mid-brain raphe (Kostowski, Giacalone, Garattini & Valzelli, 1968; Neill, Grant & Grossman, 1972). Recently, it was shown that there is a decrease in brain catecholamine turnover (Rastogi *et al.*, 1976c) along with increased synthesis and turnover of 5-HT following chronic diazepam and bromazepam treatment. This may suggest that benzodiazepines manifest their central actions by acting antagonistically on the 5-hydroxytryptaminergic and catecholaminergic neurones.

In a separate set of experiments, it has been demonstrated that chronic administration of diazepam and bromazepam decreased spontaneous locomotor activity by 61% and 49%, respectively (Rastogi *et al.*, 1976b). However, a 48 h withdrawal from these drugs significantly elevated the locomotor activity to 328% and 253%, respectively taking the values for 'treated' groups as 100%. In fact, the rats withdrawn from these minor tranquillizers displayed significantly higher mobility when compared to untreated normal rats. This is in line with the 'rebound' phenomenon observed in patients following withdrawal of anti-anxiety agents (Borland & Nicholson, 1975; Lapierre, unpublished data). It may be speculated that the elevated levels of 5-HIAA in 'withdrawn' rats could partly be due to enhanced catabolism of 5-HT within the synaptic clefts whose synthesis is decreased, but its neuronal uptake is diminished by about 30% of the values seen in diazepam- or bromazepam-treated rats (unpublished data). Additionally, the enhanced activity of MAO during withdrawal may account for lowered 5-HT and increased 5-HIAA levels seen in rats withdrawn from diazepam therapy. Since the activity of MAO did not alter significantly in rats withdrawn from bromazepam, it is conceivable that elevated levels of 5-HIAA after bromazepam withdrawal may be independent of changes in MAO activity but may be related to a reduced active transport from the brain.

In conclusion, the present study implicates central 5-hydroxytryptaminergic neurones in mediating the depressant effects of benzodiazepines on behaviour as well as in the 'rebound' phenomenon during the 'withdrawal' phase from benzodiazepine treatment as noticed earlier (Rastogi *et al.*, 1976b). However, the question whether these neurones are affected directly or are conveying the effects of another neuronal system remains open. Recent studies suggest that benzodiazepines may exert a primary action on $\gamma$-aminobutyric acid (GABA) containing neurones, some of which in turn may regulate transmission at monoaminergic synapses (Polc, Mohler & Haefely, 1974; Costa, Guidotti, Mao & Suria, 1975; Mao, Guidotti & Costa, 1975; Stein *et al.*, 1975). Padjen & Bloom (1975) suggested that acute injection of 1,4-benzodiazepines may enhance the release of GABA at 5-hydroxytryptaminergic nerve endings which by presynaptic inhibition results in a reduction of 5-HT release. However, in view of recent studies by Sherman & Gebhart (1974; 1976) demonstrating that pain induced by hot plate elevated GABA levels in the brain, it is not clear whether the increased GABA seen 1–2 h after acute administration of benzodiazepines is indeed in response to pain induced by injection or by the drug itself. Further work is necessary to gain additional insight into the effects of long-term exposure of benzodiazepines and withdrawal on GABA-containing neurones to correlate the observed changes in 5-HT metabolism with those in GABA neuronal systems. Although there is lack of evidence pertaining to the interrelationship with glycine and 5-HT metabolizing systems, the possibility remains that these anxiolytic drugs may modulate 5-HT metabolism by directly activating glycine receptors in the brain (Young, Zukin & Snyder, 1974).

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# PLASMA CONCENTRATIONS AND CARDIOTOXIC EFFECTS OF DESIPRAMINE AND PROTRIPTYLINE IN THE RAT

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1 Desipramine and protriptyline were administered to anaesthetized rats by two consecutive intravenous infusions in order to obtain a peak level (first infusion) followed by lower steady state concentrations (second infusion) (Wagner, 1974). Theoretical plasma level time courses were confirmed experimentally.

2 Desipramine and protriptyline were measured in atria and ventricles. Increasing infusion rates led to proportional increases in plasma and atrial concentrations. The tissue/medium ratio ranged from 57 to 21 for desipramine and from 43 to 11 for protriptyline according to the time of determination during infusions.

3 Heart rate changes, deviation of the electrical axis of the heart and prolongation of atrioventricular conduction were recorded at fixed times during infusion.

4 Positive chronotropic effects were noted at plasma concentrations ranging from 0.035 to 0.1 μg/ml for desipramine and from 0.04 to 1.2 μg/ml for protriptyline. At higher plasma concentrations the positive chronotropic effect decreased and bradycardia developed. Both drugs induced right rotation of the electrical axis of the heart. Threshold plasma levels giving 40° rotation were 1.35 μg/ml (desipramine) and 1.75 μg/ml (protriptyline). Atrioventricular conduction was prolonged at threshold plasma concentrations of 2.2 μg/ml for desipramine and 3.6 μg/ml for protriptyline.

5 Desipramine is more cardiotoxic than protriptyline. This difference is discussed in relation to the plasma and heart concentration of the two drugs.

## Introduction

A variety of side effects may occur during treatment with tricyclic antidepressant drugs, the most serious being the cardiac complications (Muller, Goodman & Bellet, 1961; Alexander & Nino, 1969; Coull, Crooks, Dingwall-Fordyce, Scott & Weir, 1970; Editorial 1971; Williams & Sherter, 1971). Electrocardiographic changes include sinus tachycardia, ventricular extrasystoles, prolongation of the PQ and QRS intervals and ST and T wave abnormalities.

The mechanism of action of these compounds on the heart has been attributed to the combination of their sympathomimetic, anticholinergic and membrane stabilizing effects (Sigg, Osborne & Korol, 1963; Greeff & Wagner, 1969; Schmitt, Cheymol & Gilbert, 1970; Langslet, Grini Johansen, Ryg, Skomedal & Øye, 1971; Barth & Muscholl, 1974; Elonen, Mattila & Saarnivaara, 1974). Several studies have indicated that cardiac effects are qualitatively similar among antidepressants but that from a quantitative point of view they differ widely (Boissier, Simon & Witchitz, 1965; Lechat, Auclair, Fontagné & Prudhommeau, 1969; Barth & Muscholl, 1974; Elonen *et al.*, 1974). The purpose of this study was to make a comparative investigation of the possible relationship between plasma concentrations and cardiac effects of antidepressant compounds in the rat. Since plasma levels reflect the availability of a drug at its site of action, determinations of drug concentrations in plasma may be particularly useful to find whether differences in drug activities might be explained, at least in part, by differences in pharmacokinetic behaviour. Such determinations may also make for more meaningful comparisons of drug effects among different animal species. The experimental approach chosen for this study is the theoretical model proposed by Wagner (1974) to obtain steady state plasma levels in a short period of time.

Desipramine and protriptyline were used for this comparison. These drugs are equally potent as inhibitors of catecholamine uptake in the heart

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(Carlsson & Waldeck, 1965; Franco, Bonaccorsi, Castelli, Garattini & Morselli, 1976) while there are a few reports (Boissier *et al.*, 1965) suggesting that desipramine may be a more potent cardiotoxic agent than protriptyline.

## Methods

Male Sprague Dawley rats (Charles River, Italy), weighing 200–250 g were anaesthetized with ethyl urethane (1.3 g/kg i.v.). The femoral vein and left carotid artery were cannulated and the animals were allowed to rest for 30 minutes. Desipramine (DMI) and protriptyline (PTP) were administered by two consecutive intravenous infusions. The first infusion lasted 10 min ($Q_1$) and was followed by a second one ($Q_2$), by which time steady state plasma levels were reached and maintained. Wagner equations (1974) were used to calculate the infusion rates. The necessary pharmacokinetic parameters were obtained from the disappearance curves of DMI and PTP from plasma of rats given an intravenous injection of 5 mg/kg of the drugs (Table 1). Calculations were made with a Digital PDP 11/45 computer fed the following information: (i) pharmacokinetic parameters; (ii) duration of the first infusion; (iii) the desired steady state plasma level. Table 2 shows the infusion rates in µg $kg^{-1}$ $min^{-1}$ and the theoretical plasma levels. The intravenous infusions of DMI and PTP were delivered by a constant infusion pump (Braun, Germany) at the rate of 0.05 ml/minute. Solutions were warmed to 37°C just before entering the femoral vein. Control animals were infused with 0.9% w/v NaCl solution (saline). During the infusions, the three bipolar standard limb leads of the ECG were recorded on

**Table 1** Pharmacokinetic parameters of desipramine (DMI) and protriptyline (PTP) calculated from single bolus intravenous injections (5 mg/kg) in anaesthetized rats according to a two compartment open model

| *Parameters* | *Desipramine* | | *Protriptyline* | |
|---|---|---|---|---|
| *K*el | 0.0152 | $min^{-1}$ | 0 0128 | $min^{-1}$ |
| *K*12 | 0 0873 | $min^{-1}$ | 0 0986 | $min^{-1}$ |
| *K*21 | 0.0503 | $min^{-1}$ | 0.0349 | $min^{-1}$ |
| $\alpha$ | 0 14296 | $min^{-1}$ | 0 14762 | $min^{-1}$ |
| $\beta$ | 0.00301 | $min^{-1}$ | 0 00518 | $min^{-1}$ |
| V1 | 4 956 | l/kg | 5.31 | l/kg |
| $(T_{\frac{1}{2}})\beta$ | 230 41 | min | 133 8 | min |
| TBCl | 0 06093 | l $kg^{-1}$ $min^{-1}$ | 0.08078 | l $kg^{-1}$ $min^{-1}$ |

Kel = first order elimination rate constant; K12 and K21 = first order distribution rate constants between the two compartments; $\alpha$ and $\beta$ = disposition rate constant; V1 = apparent volume of the central compartment; $(T_{\frac{1}{2}})\beta$ = apparent plasma half life of the $\beta$ phase; TBCl = Total body clearance.
The infusion rates in Table 2 were calculated by Wagner's equation (1974) from these data

**Table 2** Desipramine (DMI) and protriptyline (PTP) infusion rates and theoretical steady state plasma levels (µg/ml)

| *Desipramine* | | | *Protriptyline* | | |
|---|---|---|---|---|---|
| *Rates of infusion** *(µg $kg^{-1}$ $min^{-1}$)* | | *Theoretical steady state plasma levels (µg/ml)* | *Rates of infusion** *(µg $kg^{-1}$ $min^{-1}$)* | | *Theoretical steady state plasma levels (µg/ml)* |
| 1/10.Q | $Q_1$=63.6<br>$Q_2$= 3.6 | 0 035 | 1/10 Q | $Q_1$=57.3<br>$Q_2$= 5.7 | 0.040 |
| 1.Q | $Q_1$=636.6<br>$Q_2$= 36.3 | 0 35 | 1.Q | $Q_1$=573.5<br>$Q_2$= 57.5 | 0.40 |
| 2.Q | $Q_1$=1273.3<br>$Q_2$=72.6 | 0.70 | 2.Q | $Q_1$=1147<br>$Q_2$= 115 | 0.80 |
| 3.Q | $Q_1$=1909.9<br>$Q_2$=108.9 | 1.05 | 3.Q | $Q_1$=1720<br>$Q_2$= 172 | 1 20 |
| 5.Q | $Q_1$=3183.2<br>$Q_2$=181.5 | 1.75 | 5.Q | $Q_1$=2867<br>$Q_2$= 287.5 | 2.00 |

* $Q_1$ =first infusion given for 10 min; $Q_2$=infusion maintaining steady state.

OTE Biomedica equipment through subcutaneous needle electrodes. Sensitivity was adjusted so that 1 mV was equal to 1.5 cm. Heart rate and PQ interval were measured graphically. The position of the electrical axis was determined by the procedure of Goldman (1970).

In some experiments, at different times after the start of drug administration, blood was collected (about 5 ml) in heparinized tubes from the carotid artery, and centrifuged for 30 min at 2000 rev/min and 4°C.

The hearts were removed, atria and ventricles were separated, blotted with filter paper and weighed. All specimens were kept frozen until analysis.

### *Electron capture gas chromatographic determination of desipramine and protriptyline in tissue samples*

Atria and ventricles were homogenized with glass homogenizers in 2.5 and 0.1 N HCl respectively.

DMI and PTP concentrations were determined by gas chromatography, following a modified version of the method described by Borgå & Garle (1972). Tissue and plasma samples (0.1–1 ml) were added with phosphate buffer (pH 11, 0.3 M) to a final volume of 2 ml. As an internal marker 4 μg of maproptyline was added to samples containing DMI and 0.8 μg to each sample of PTP. Drugs were extracted with 5 ml of ethyl ether and, after discarding the aqueous phase, re-extracted in 2.5 ml of 0.1 N HCl. The acidic aqueous phase was made alkaline with 0.2 ml of 2 N NaOH and again extracted with 5 ml of ethyl ether. After centrifugation, 4.5 ml of the ether phase were transferred to a test tube and evaporated to dryness at 40°C in a water bath. Heptafluorobutyric anhydride (200 μl of 1 : 10 solution in ethyl acetate) was added to the drug residue. The solution was shaken in glass capped tubes, incubated at 40°C for 30 min and then evaporated to dryness under a stream of $N_2$ at 40°C for 10 minutes. Samples were dissolved in 0.5 ml ethyl ether, shaken and evaporated again to dryness at 40°C under a stream of $N_2$ for 15 minutes. The derivative was dissolved in 1 ml (DMI samples) or 300 μl (PTP samples) of ethyl acetate; 1 μl of these solutions was injected into the gas chromatograph. Overall recovery was 75 ± 3% for both drugs. A Carlo Erba Fractovap G-1 gas chromatograph equipped with a $^{63}$Ni electron capture detector was used. The chromatographic column was a glass tube, 1 m long, 4 mm i.d., packed with 100–200 mesh Gas Chrom Q, coated with 3% OV 17 (Applied Science Laboratories) treated as previously described (Di Salle, Baker Bareggi, Watkins, Chidsey, Frigerio & Morselli, 1973). The operating conditions were: column temperature 230°C; detector temperature 275°C; carrier gas ($N_2$) flow rate 60 ml/min, scavenger gas ($N_2$) flow rate 70 ml/minute. The electron capture detector pulse interval was 30 milliseconds. For both compounds the calibration curves (constructed from the ratio of the peak area for the test compound to that of the internal marker) were linear from 60 to 900 ng/sample. Minimum sensitivity was 15 ng/sample for DMI and 60 ng/sample for PTP.

### *Drugs and reagents*

The following drugs were used: desipramine hydrochloride and maproptyline (34276 Ba) (Ciba Geigy, Basel, Switzerland and Ciba Geigy, Milan, Italy); protritpyline hydrochloride (Merck Sharp & Dohme, Rahway, N.J., USA). All drug concentrations given in the present study are expressed as free bases. Ethyl urethane and the solvents (analytical grade) for determination of desipramine and protriptyline were purchased from Carlo Erba (Milan, Italy). Heptafluorobutyric anhydride was purchased from Fluka (Switzerland).

## Results

### *Desipramine and protriptyline concentrations in rat plasma and heart*

The theoretical time courses of DMI and PTP obtained by Wagner's method (1974) follow the continuous lines in Figure 1. Simulated values were confirmed experimentally by measuring plasma levels in the rat during 1.Q infusion (see Table 2) at 10, 40 and 70 minutes. Drug concentrations stabilized at a steady state during the second infusion after reaching peak levels at the end of the loading infusion. Plasma levels increased proportionally as the infusion rate was raised from 1.Q to 5.Q.

In the same experiments DMI and PTP were measured in atria and ventricles, where they accumulated extensively. Both drugs showed similar tissue to plasma ratios after 10 min of infusion, while at 40 and 70 min PTP tissue levels declined faster than those of DMI (Table 3). Higher plasma levels caused a proportional increase in the atria and ventricles (Figure 2).

### *Effects of desipramine and protriptyline on the ECG tracing of the rat: correlation with plasma-heart levels*

As shown in Figure 3 heart rate increased at DMI plasma levels of 0.035 and 0.10 μg/ml; at plasma levels of 0.35, 0.70 and 1.05 μg/ml (steady state values) no changes in heart rate were detected; further increase in the plasma concentration from 1.0 to 4.0 μg/ml (peak levels), produced progressively greater bradycardia.

With PTP the tachycardia was considerably greater than with DMI (plasma concentrations from 0.04 to 1.2 μg/ml). Only following a peak level of 6 μg/ml did

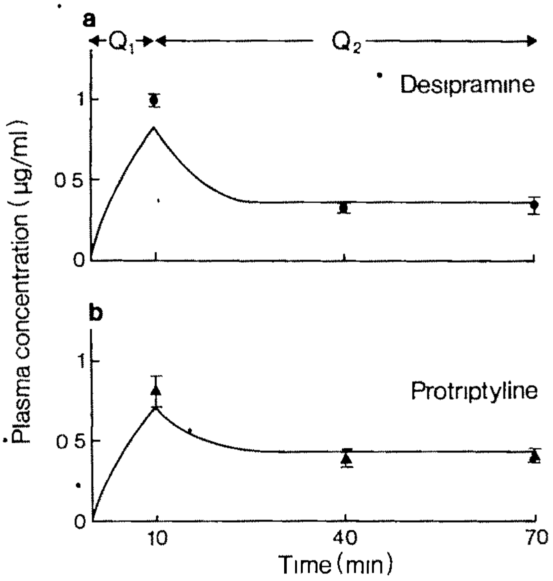

**Figure 1** Time course of theoretical and experimental plasma concentrations of (a) desipramine and (b) protriptyline in the rat after two consecutive infusions of the drugs at the rate 1.Q (see Table 2). An initial infusion was given for 10 min then the rate was lowered and maintained for 70 minutes. The continuous line was drawn by a computer while experimental values were measured at 10, 40 and 70 min of infusion. Each point is the average of 6 (at 10 min) or 3 (at 40 and 70 min) determinations. Vertical lines represent s.e. mean.

PTP produce bradycardia. However, a depressant component became superimposed on the stimulation at peak plasma concentrations of 2.0 and 3.5 μg/ml.

Data obtained after 10 min of infusion (plasma concentrations range from 0.79 to 6.0 μg/ml) showed that cardiac alterations were all significantly correlated with plasma and heart levels. Figure 4 shows the relationship between heart rate changes and the concentrations of DMI and PTP in plasma and atria. PTP produces tachycardia at concentrations that are depressant for DMI and this difference persists with both plasma and atrial concentrations. During the steady state period (plasma concentrations range from 0.35 to 2.0 μg/ml) heart rate changes did not correlate with the corresponding plasma levels.

Besides the effect on the heart rate, DMI and PTP induced morphological alterations of the QRS complex, consisting of decreased voltage amplitude of the R wave and greater depth of the S wave, especially in lead 1. These alterations, which are expressed by a right rotation of the electrical axis of the heart, are plasma-level-dependent, and are more marked for DMI than for PTP (Figure 5, above).

**Table 3** Desipramine (DMI) and protriptyline (PTP) concentrations in plasma, atria and ventricles during two consecutive infusions

| *Compound* | *Time (min)* | *Plasma levels (μg/ml)* | *Atria levels (μg/g)* | *Ventricle levels (μg/g)* | *Atria/plasma* | *Ventricles/plasma* |
|---|---|---|---|---|---|---|
| Desipramine 1.Q* | 10 | 1.0±0.09 | 37.4±3 | 43 77±8 | 37.4±3 | 43.7±2 |
| | 40 | 0.32±0.021 | 15.6±2 | 10.1±2 | 48.2±7 | 30.7±4 |
| | 70 | 0.30±0.025 | 17.3±1 | 11.5±1 | 57 4±5 | 38.5±4 |
| Protriptyline 1.Q* | 10 | 0.79±0.053 | 27.9±2 | 30.9±0.9 | 32.2±3 | 37.7±5 |
| | 40 | 0.37±0.014 | 9.9±0.6 | 5.8±0.7 | 27 1±2 | 18.0±2 |
| | 70 | 0.39±0.047 | 8.1±1 | 4.2±0.4 | 21.0±2 | 11.5±2 |

* The infusion rates are shown in Table 2.
All the results are the average of 6 (10 min) or 3 (40 and 70 min) determinations.

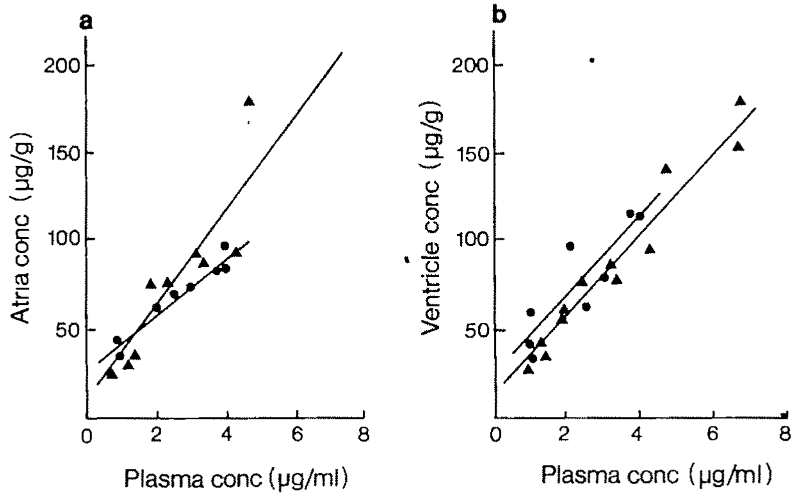

**Figure 2** Correlation between plasma concentrations and (a) atrial or (b) ventricular concentrations of desipramine (●) and protriptyline (▲). All determinations were made at the end of the first infusion. The infusion rate ranged from 1 Q to 5 Q (see Table 2). (a) For desipramine $r=0.98$, $P<0.001$; for protriptyline $r=0.96$, $P<0.001$, (b) for desipramine $r=0.87$, $P<0.01$; for protriptyline, $r=0.98$, $P<0.01$

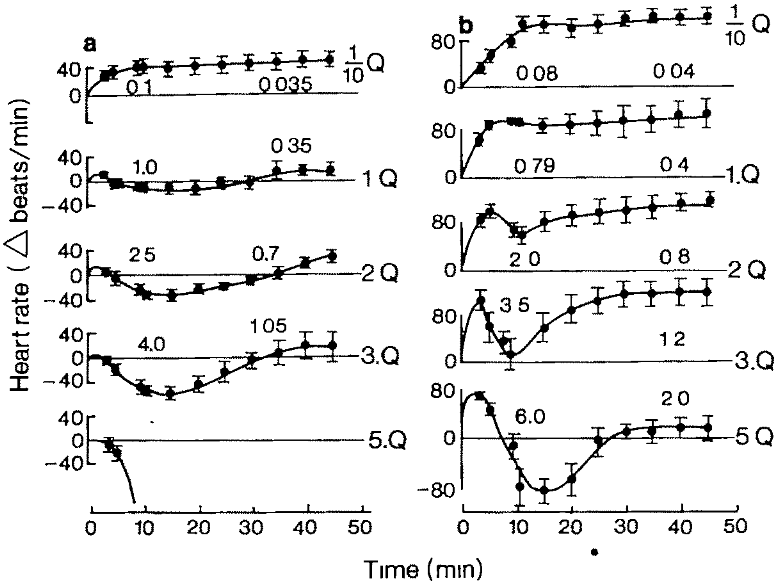

**Figure 3** Time course of changes in heart rate produced by (a) desipramine and (b) protriptyline in anaesthetized rats given the two drugs at different infusion rates. 1.Q (and multiples) indicate infusion rates (set out in Table 2). Numbers inside the graph indicate the plasma levels of the drugs in µg/ml at 10 and 40 min during infusions. Groups of 4 or 5 rats were used for each infusion rate. Vertical lines represent s.e. mean.

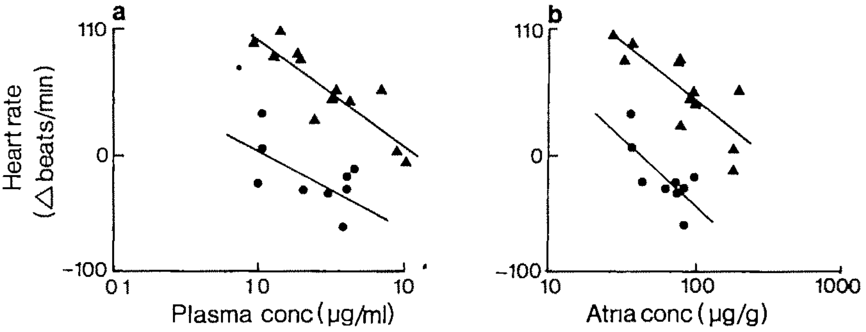

Figure 4 Correlation between (a) plasma or (b) atrial concentrations and changes in heart rate induced by desipramine (●) and protriptyline (▲). Values were obtained after 10 min infusion at rates ranging from 1.Q to 5.Q (set out in Table 2) The statistical significance of these correlations is given in Table 4.

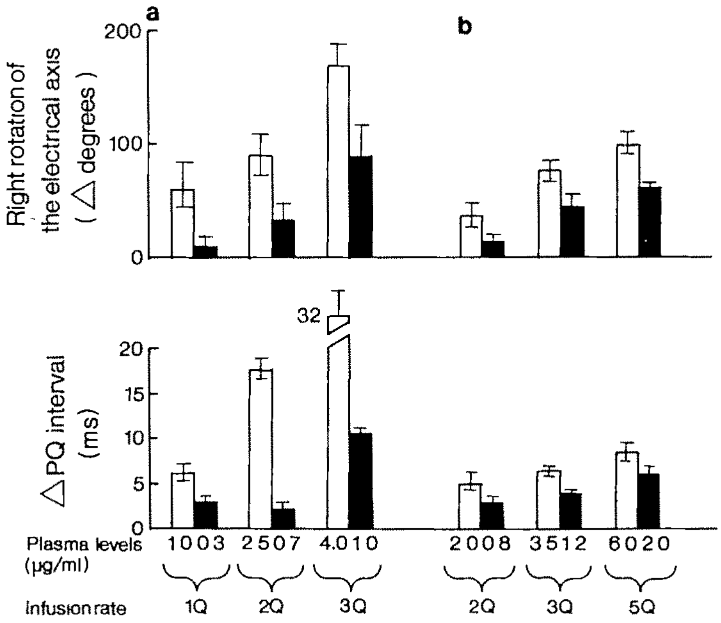

Figure 5 Effect of (a) desipramine and (b) protriptyline on the electrical axis of the heart (above) and the PQ interval (below) in anaesthetized rats given the two drugs at different infusion rates. 1.Q and multiples indicate the infusion rates (set out in Table 2). Plasma concentrations are also shown in µg/ml. Determinations were made at 10 min (open columns) and 40 min (solid columns) during the two consecutive infusions. Desipramine and protriptyline induced a right rotation of the electrical axis of the heart and prolongation of the PQ interval, expressed as the difference from preinfusion values. Groups of 4 or 5 rats were used for each infusion rate. Vertical lines represent s.e. mean.

DMI and, at the highest infusion rates, PTP, slowed atrioventricular conduction. The lengthening of the PQ interval was also plasma-level-dependent (Figure 5, below).

The *r* of correlation and *P* of significance of the relationship between plasma levels and cardiac alterations are shown in Table 4.

From the data obtained during the first 10 min of infusion, at different rates the plasma concentrations capable of inducing given ECG changes were determined. Forty degrees rotation of the electrical axis was caused by 1.35 µg/ml DMI and 1.75 µg/ml

PTP, while 2.2 µg/ml DMI and 3.6 µg/ml PTP lengthened the PQ interval by 20%.

DMI at plasma concentrations of 4 µg/ml caused marked alterations of the ECG tracing, i.e. ectopic beats, bigeminy, partial or complete atrioventricular blockade and repolarization disturbances. This concentration greatly depressed respiration, although this does not appear to be the direct cause of death because animals with or without artificial respiration died within the same time and had similar ECG alterations.

## Discussion

The aim of this study was to develop a suitable model for testing the cardiotoxicity of tricyclic antidepressant agents, a severe side effect which limits the use of these compounds in clinical practice. The study was carried out in rats by mimicking, according to the model proposed by Wagner (1974), the situation that arises during repeated therapeutic treatment in man, i.e. an absorption peak after each drug intake followed by a steady state plasma concentration (Alexanderson, 1972; Garattini & Morselli, 1975).

Two widely used tricyclic antidepressant agents, desipramine (DMI) and protriptyline (PTP) were studied. The selected plasma levels, ranging from 0.035 to 6 µg/ml, comprised both therapeutic plasma levels (25 to 700 ng/ml according to Yates, Todrick & Tait, 1963; Åsberg, Crönholm, Sijöqvist & Tuck, 1971; Åsberg, 1974; Garattini & Morselli, 1975) and those found after an accidental overdose (600 to 2190 ng/ml: Spiker, Weiss, Chang, Ruwitch & Biggs, 1975).

In agreement with *in vitro* findings (Babulova, Bareggi, Bonaccorsi, Garattini, Morselli & Pantarotto, 1973; Franco *et al.*, 1976) tricyclic antidepressant agents accumulate in heart tissue at ratios of 21 and 57 for DMI and 11 and 43 for PTP. These ratios are lower than those reported for PTP in the rabbit by Elonen, Linnoila, Lukkari & Mattila (1975) but are comparable to those obtained by Curry (1964) and Rasmussen (1965) in human *post mortem* samples.

The constant ratio between heart and plasma levels indicates that plasma levels are highly predictive of the concentration of these drugs in heart tissue.

The cardiac effects of DMI and PTP analyzed were: (i) changes in the heart rate; (ii) right rotation of the electrical axis (Boissier *et al.*, 1965; Lechat *et al.*, 1969) and (iii) impairment of atrioventricular conduction shown by prolongation of the PQ interval (Fekete & Borsy, 1964; Baum, Shropshire, Rowles & Gluckman, 1971; Elonen *et al.*, 1974).

Both DMI and PTP caused a two-phase effect on heart rate. Relatively low plasma concentrations of both drugs increased the heart rate while higher plasma concentrations reduced it.

**Table 4** Statistical analysis of the relationships between plasma concentrations of desipramine (DMI) and protriptyline (PTP) and their cardiac effects

| *Relationship between plasma levels and changes in.* | *Time of infusion (min)* | *Desipramine* | | | *Protriptyline* | | |
|---|---|---|---|---|---|---|---|
| | | *No. of rats* | r | P | *No. of rats* | r | P |
| Heart rate | 10 | 8 | −0.68 | <0.05 | 10 | −0.77 | <0.01 |
| Electrical axis position | | 7 | +0.84 | <0.02 | 10 | +0.87 | <0.01 |
| PQ interval | | 7 | +0.7 | <0.05 | — | — | — |
| Heart rate | 40 | 14 | −0.1 | >0.05 | 12 | −0.15 | >0.05 |
| Electrical axis position | | 14 | +0.70 | <0.05 | 12 | +0.82 | <0.05 |
| PQ interval | | — | — | — | — | — | — |

The increase in the heart rate attributed to anticholinergic effects and to inhibition of catecholamine uptake (Caincross & Gershon, 1962; Editorial, 1971) does not seem to be related to plasma levels. Tachycardia following tricyclic antidepressants is also frequent in man within the therapeutic range (Ayd, 1968; Editorial, 1971).

The depressant effect on heart rate, probably due to a membrane stabilizing effect (Greff & Wagner, 1969; Schmitt *et al.*, 1970; Marmo, Coscia & Cataldi, 1972; Elonen *et al.*, 1974), was directly related to peak plasma concentrations for both drugs. The lack of correlation during the steady state might be due to low plasma levels producing only tachycardia (PTP) or a combination of positive and negative chronotropic effects resulting in no change of the heart rate (DMI).

Another effect common to both DMI and PTP is the right rotation of the electrical axis of the heart. The threshold plasma concentrations for this effect were found to be 1.35 and 1.75 µg/ml for DMI and PTP respectively, and the intensity was directly related to plasma concentrations.

Finally, impaired atrioventricular conduction (prolongation of the PQ interval) was noted at a level of 2.2 µg/ml for DMI and 3.6 µg/ml for PTP. Impaired atrioventricular conduction was observed in man only following drug overdosage by Vohra (1974), Thorstrand (1974) and by Spiker *et al.* (1975).

Our results indicate that DMI and PTP affect the AV conduction at plasma concentrations above the therapeutic levels. However, the threshold concentrations of DMI and PTP inducing cardiac alterations might be substantially lowered if cardiac function is already impaired or in the presence of other stimuli. Although this suggestion has been made by several authors (Jefferson, 1975) there are other reports with no evidence to support this suggestion (Report from Boston Collaborative Drug Surveillance Program, 1972).

In this investigation and within the limits of the plasma concentrations tested, DMI appeared to be more cardiotoxic than PTP. Although at comparable peak plasma levels, similar concentrations of the two drugs were found in the heart, during the steady state twice as much DMI accumulates as PTP (Table 4). Therefore, as well as its different intrinsic activity, different availability to cardiac tissue may also contribute to making DMI more cardiotoxic than PTP.

The experimental model presented here may be of interest for exploring the cardiotoxicity of other tricyclic antidepressants *in vivo* and for studying factors which may aggravate or inhibit this toxicity. The results obtained could also be of clinical importance since extrapolation is facilitated by the fact that the toxic doses of tricyclic antidepressant drugs are expressed in terms of peak or steady state plasma concentrations.

R.G. was an IBM Italia Fellow.

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# PLASMA NORTRIPTYLINE AND CARDIAC RESPONSES IN YOUNG AND OLD RATS

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1 The relationship between plasma concentrations and cardiac effects of nortriptyline was studied in anaesthetized young and old rats.

2 Nortriptyline was administered by two consecutive intravenous infusions which resulted in a peak plasma concentration followed by steady state values. Increasing infusion rates were followed by proportional increases in the drug plasma concentrations ranging from 0.15 to 6.0 μg/ml.

3 In young rats, nortriptyline induced an increase in the heart rate, a right rotation of the electrical axis and a prolongation of the PQ interval. Heart rate changes were not correlated with nortriptyline plasma concentrations, while significant correlations were found for the other two parameters. Plasma concentrations inducing 20% increase of the PQ interval and 40 degrees rotation of the electrical axis were 1.65 and 1.69 μg/ml respectively. Arrhythmias occurred at concentrations higher than 5.2 μg/ml.

4 Nortriptyline caused more severe cardiac effects in old than in young animals. However, plasma concentrations of nortriptyline in old rats were two to five times higher than those found in young rats at similar infusion rates. A higher concentration of the drug at its sites of action seems to be responsible for the more severe cardiac toxicity of nortriptyline observed in old rats.

## Introduction

The cardiac effects of tricyclic antidepressant agents have been well documented both in cases of accidental overdosage (Vohra, 1974; Thorstrand, 1974) as well as during chronic therapy (Coull, Crooks, Dingwall-Fordyce, Scott & Weir, 1970; Editorial 1971). ECG changes include tachycardia, arrhythmias, widening of the PQ and QRS complex, ST and T wave abnormalities. It has also been shown that antidepressant drugs aggravate cardiac disease especially in elderly people suffering from hypertension and atherosclerosis (Muller, Goodman & Bellet, 1961; Kristiansen, 1961; Skou, 1962; Thompson, 1973).

According to some authors (Alexanderson & Sjöqvist, 1971; Spiker, Weiss, Chang, Ruwitch & Biggs, 1975) there is a positive correlation between cardiac side effects and plasma concentrations of the drugs in humans. ECG alterations were also observed in laboratory animals treated with antidepressant drugs. In the rat, desipramine and protriptyline prolong the PQ interval, depress the heart rate and cause rotation to the right of the heart electrical axis. All these effects are dependent on the plasma concentrations of the drugs (Bianchetti, Bonaccorsi, Chiodaroli, Franco, Garattini, Gomeni & Morselli, 1977).

The present paper examines the possibility that nortriptyline may be a more cardiotoxic agent in the old than in the young rat. Well defined cardiac effects were related to plasma concentrations in both groups of animals in order to check whether a more marked cardiotoxicity in old animals could be attributed to altered sensitivity to the drug, or to greater availability of the drug at its sites of action.

## Methods

### Experimental procedure

Young (7 weeks old, 200–250 g body wt.) and old (2 years old, 700–900 g body wt.) rats supplied by Charles River (Italy) were anaesthetized with ethyl urethane at doses of 1.3 and 1.0 g/kg respectively. The femoral vein and left carotid artery were cannulated and the animals were allowed to rest for 30 minutes. Nortriptyline was administered by two consecutive intravenous infusions. The first infusion lasted 10 min ($Q_1$) and was followed by a second one ($Q_2$) at a

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**Table 1** Pharmacokinetic parameters of nortriptyline calculated from the disappearance curve of the drug after intravenous injection of 5 mg/kg in anaesthetized young rats. The disappearance curve fitted a two compartment open model

| | |
|---|---|
| Kel | 0.03306 $min^{-1}$ |
| $K_{12}$ | 0.1805 $min^{-1}$ |
| $K_{21}$ | 0.1119 $min^{-1}$ |
| $\alpha$ | 0.31366 $min^{-1}$ |
| $\beta$ | 0.01179 $min^{-1}$ |
| $V_1$ | 4.2918 1/kg |
| $(T_{\frac{1}{2}})_\beta$ | 58.78 min |
| TBCl | 0.13769 1/kg min |

Kel: first order elmination rate constant; $K_{12}$ and $K_{21}$: first order distribution rate constants between the two compartments; $\alpha$ and $\beta$: disposition rate constants; $V_1$: apparent volume of the central compartment. $(T_{\frac{1}{2}})_\beta$: apparent half-life of the $\beta$-phase; TBCl: total body clearance.

The infusion rate 1.Q calculated from Wagner's equation (1974) was as follows: 369 µg/kg for the first 10 min ($Q_1$); 35 µg/kg for the rest of the infusion ($Q_2$). The other rates were multiples of 1.Q.

slower rate, by which steady state plasma levels were reached and maintained. The pharmacokinetic parameters obtained after a bolus injection of nortriptyline (see Table 1) were used to calculate the infusion rates according to Wagner's equations (1974). The infusion rates ranges from 1/3 ($Q_1 = 121$ µg $kg^{-1}$ $min^{-1}$ and $Q_2 = 11.6$ µg $kg^{-1}$ $min^{-1}$) to 8 times Q ($Q_1 = 2952$ µg $kg^{-1}$ $min^{-1}$ and $Q_2 = 280$ µg $kg^{-1}$ $min^{-1}$). A Digital PDP 11/40 computer was used for calculations. The intravenous infusions of nortriptyline were delivered by a constant infusion pump (Braun, Germany) at the rate of 0.05 ml/minute. Because of the poor solubility of the drug in water the rate was increased to 0.1 or 0.2 ml/min for the highest concentrations of nortriptyline. Solutions were heated to 37°C just before entering the femoral vein. During infusions, the three bipolar standard limb leads of the electrocardiogram were recorded on Ote Biomedica equipment through subcutaneous needle electrodes. Sensitivity was adjusted so that 1 mV was equal to 1.5 cm.

Heart rate and PQ interval were measured at fixed intervals. QRS and QT intervals could not be quantified since the lack of isoelectric tract and the difficulty in determining the end of the T wave in the rat ECG complex do not permit accurate measurements of these parameters. The position of the electrical axis of the heart was determined following the procedure described by Goldman (1970).

Blood was collected from the cannulated carotid artery in heparinized tubes and centrifuged for 30 min at 2000 rev/min at 4°C. Three samples of 0.5 ml were withdrawn from old animals, at 10, 30 and 60 min after starting infusion. From young rats, blood was collected only once at the end of the experiment. When rats were killed their hearts were removed, blotted with filter paper and weighed. All specimens were kept frozen until analysis.

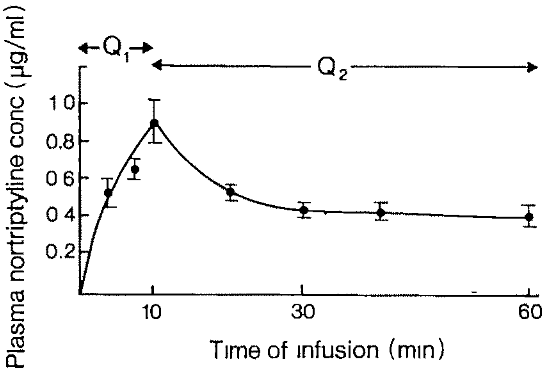

**Figure 1** Time course plasma levels of nortriptyline in young rats given two consecutive infusions ($Q_1$ and $Q_2$) of the drug at the rate 1.Q (see Table 1 for corresponding values). Each point is the average of 3–5 determinations. Vertical lines show s.e.

### *Gas-chromatographic determination of nortriptyline in tissue samples*

Atria and ventricles were homogenized with glass homogenizers in 2.5 and 8 ml 0.1 N HCl respectively. Nortriptyline in biological samples was quantitatively determined by electroncapture gas-chromatography following the procedure described for desipramine and protriptyline (Bianchetti *et al.*, 1977). The overall recovery was 75%. The calibration curve, constructed from the ratios of the peak areas of the test compound to those of the internal marker maproptyline, was linear from 50 to 400 ng/sample. Minimal sensitivity was 50 ng/sample.

### *Drugs and reagents*

Nortriptyline hydrochloride was supplied by Recordati, Milan, Italy; maprotyline (34276 Ba) was supplied by Ciba Geigy, Basel, Switzerland. All drug concentrations are expressed as free bases. Ethyl urethane and the solvents (analytical grade) used for nortriptyline determinations were purchased from Carlo Erba, Milan, Italy.

## Results

### *Nortriptyline plasma and heart concentrations in young and old rats*

The time course of the plasma levels of nortriptyline given to young rats by two consecutive infusions at the rate 1.Q is shown in Figure 1. Plasma levels progressively increased during the first infusion ($Q_1$), then rapidly declined and stabilized at a steady state during the second infusion ($Q_2$).

When infusion rates were decreased to 1/3.Q or increased up to 8.Q, plasma levels at 10 and 60 min increased proportionally (Table 2). Heart concentrations measured at the same times showed that nortriptyline was taken up in the heart by a factor of 12–23 times compared to the plasma concentrations.

Old rats were given nortriptyline at the infusion rate 2.Q and 3.Q. Their plasma concentrations were determined at 10, 30 and 60 min from the beginning of infusion. Table 3 shows the plasma and heart levels found in each animal. At both infusion rates the concentrations of the drug in plasma and heart were three to five times higher than in young rats. Four rats out out of seven survived after the first 10 min of infusion. In these animals, after a very high peak, plasma concentrations rapidly declined within 30 min of infusion, then slightly increased with time till 60 minutes. Nortriptyline uptake by the heart of old rats was not different from that in the heart of young rats, as indicated by similar heart/plasma ratios in both groups of rats.

*Cardiac effects of nortriptyline in young rats*

In young rats nortriptyline infusions induced marked changes in the ECG tracing. Some of these alterations, i.e. heart rate changes, rotation of the electrical axis to the right and prolongation of the PQ interval, were plotted with the corresponding plasma concentrations and tested for correlation at 10 and 60 min of infusion. Except for a mild increase in heart rate (+23 beats/min after 60 min), no alterations to the ECG tracing were induced by saline infusion.

In most of the animals nortriptyline increased the heart rate at plasma concentrations ranging from 0.1 to 6.0 µg/ml. This increase was greater after 60 min than after 10 min of infusion, and was not plasma concentration-dependent (Figure 2).

The right rotation of the electrical axis and the prolongation of the PQ interval induced by nortriptyline were significantly correlated with plasma concentrations. Figures 3 and 4 show that the slopes of the regression lines after 60 min of infusion are significantly less steep than those obtained after 10 minutes. The threshold plasma concentrations of nortriptyline causing 40 degrees rotation of the electrical axis and a 20% increase of the PQ interval,

**Table 2** Plasma and heart concentrations of nortriptyline in young rats at 10 and 60 min during nortriptyline infusions

| *Infusion rates* | *Plasma (µg/ml)* *10 min* | *Plasma (µg/ml)* *60 min* | *Heart (µg/ml)* *10 min* | *Heart (µg/ml)* *60 min* | *Heart/plasma ratio* *10 min* | *Heart/plasma ratio* *60 min* |
|---|---|---|---|---|---|---|
| 1/3.Q | 0.32±0.06 | 0.15±0.01 | 5.73±0.27 | 1.91±0.33 | 19.0±4.0 | 12.6±2.5 |
| 1.Q | 0.90±0.12 | 0.40±0.02 | 12.92±0.65 | 5.02±0.63 | 15.5±2.4 | 12.8±2.0 |
| 2.Q | 1.71±0.16 | 0.86±0.07 | 24.81±1.00 | 13 39±1.62 | 14.7±1.5 | 17.0±3.2 |
| 3.Q | 2.26±0.16 | 1 35±0.40 | 38.69±3.26 | 21.28±1.80 | 17.2±1 7 | 16.0±1.0 |
| 4.Q | 3.02±0.18 | 1.70±0.18 | 63.84±8.44 | 26.52±2.77 | 21.2±3 9 | 16.5±2.0 |
| 5.Q | 3.35±0.31 | 2.57±0.20 | 74.88±5.92 | 32 34±2.24 | 23.2±2.5 | 12.0±1.7 |
| 7.Q | 5.20±0.50 | 3.30±0.28 | not measured | | – | – |
| 8.Q | 6.0 ±0.55 | 3.70±0.31 | not measured | | – | – |

**Table 3** Individual plasma and heart concentrations of nortriptyline in old rats at 10, 30 and 60 min during nortriptyline infusion

| *Infusion rates* | *Rat No.* | *Plasma (µg/ml)* *10 min* | *Plasma (µg/ml)* *30 min* | *Plasma (µg/ml)* *60 min* | *Heart (µg/g)* *10 min* | *Heart (µg/g)* *60 min* | *Heart/plasma* *10 min* | *Heart/plasma* *60 min* |
|---|---|---|---|---|---|---|---|---|
| 2.Q | 1 | 5.30 | 1.20 | n.d. | | | | |
| | 2 | 4.50 | 1.05 | 1.70 | | 26.7 | | 15.7 |
| | 3 | 5.85 | 1.65 | 2.25 | | 53.7 | | 22 |
| | 4 | 6.75 | 2.05 | 2.65 | | 45.6 | | 17 |
| 3.Q | 5 | 11.10 | died at 10 min | | 121.8 | | 11 | |
| | 6 | 8.40 | died at 10 min | | 222.1 | | 26 | |
| | 7 | 9.45 | died at 10 min | | 177.0 | | 18.7 | |

Rat no. 3 showed AV dissociation and arrhythmias before nortriptyline infusion.
n.d. = not measured.

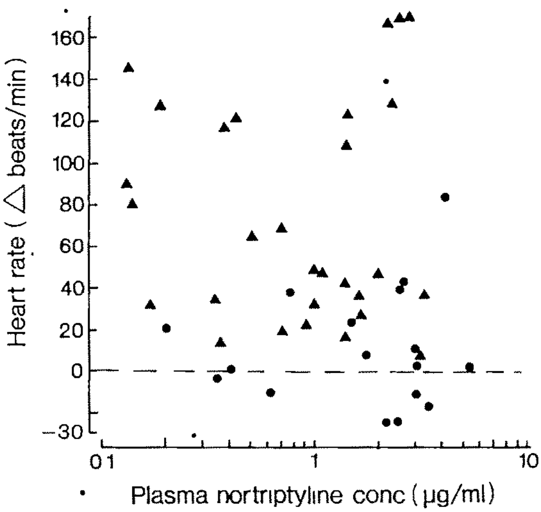

**Figure 2** Relationship between plasma concentrations of nortriptyline (abscissa scale) and changes in heart rate (ordinate scale) in young rats at 10 (●) and 60 (▲) min of nortriptyline infusions given at the rates 1/3.Q–8.Q. Regression coefficient for (●)=0 108 and for (▲)=0.103; $P>0.05$ for both.

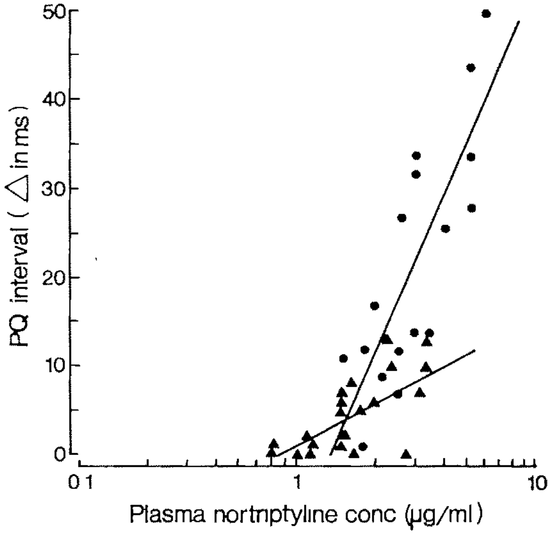

**Figure 3** Relationship between plasma concentrations of nortriptyline (abscissa scale) and the shift to the right of the electrical axis of the heart (ordinate scale) in young rats at 10 (●) and 60 (▲) min of nortriptyline infusions given at the rates 2.Q–8.Q. Preinfusion electrical axis values were 60°. The correlation lines are significantly different: for (●) $r$=0.73, $P<0.01$, a=−979 (490–1469), b=311 (167–455); for (▲) $r$=0.61, $P<0.01$, a=−275 (100–451), b=44 (39–149).

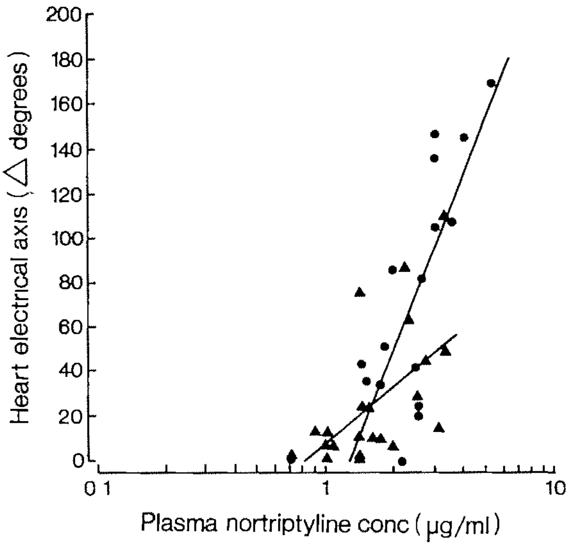

**Figure 4** Relationship between plasma concentrations of nortriptyline (abscissa scale) and the prolongation of the PQ interval (ordinate scale) in young rats at 10 (●) and 60 (▲) min of nortriptyline infusion given at the rates 2.Q–8.Q. Preinfusion PQ interval values were 54 7 ± 4 ms. The two correlation lines are significantly different: for (●) $r$=0.84, $P<0.001$, a=−204 (132–276), b=64 (22–43); for (▲) $r$=0.70, $P<0.01$, a=−44 (21–67), b=15 (4–11).

calculated from the equation of the regression lines (10 min), were 1.65 and 1.69 µg/ml respectively. A significant correlation between these two last ECG alterations and heart nortriptyline levels was also found as might have been expected from the constant ratio of nortriptyline concentrations in tissue and plasma.

Arrhythmias (extrasystoles, bigeminism, AV dissociation and ventricular tachycardia) occurred in young rats at peak plasma levels higher than 5.2 µg/ml at the infusion rate 7.Q and 8.Q.

### *Cardiac effects of nortriptyline in old rats*

The basal ECG tracing of old rats showed some characteristics worthy of mention. The heart of these animals (246 beats/min) was slower than in young animals (314 beats/minute). Hypertrophy of the left ventricle in old animals was indicated by the position of the electrical axis (between 0° and 30°) and then confirmed by postmortem anatomical examination. Atrioventricular conduction was not significantly different in young (54.75 ± 4 ms) and old rats (61.6 ± 6 ms) while the QRS complex in three out of seven old rats was widened. Atrioventricular dissociated and extrasystoles were noted in the ECG tracing of one old rat out of seven.

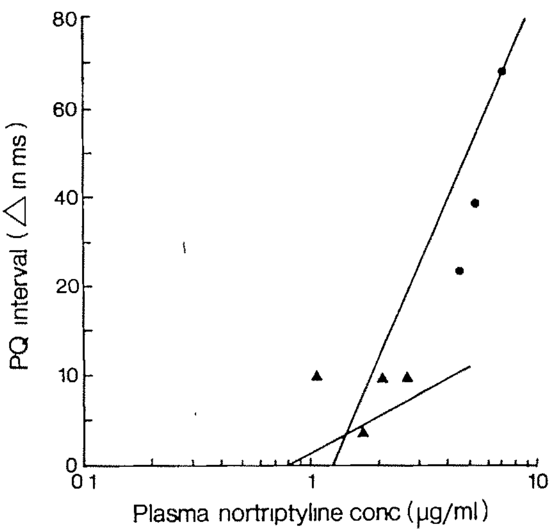

**Figure 5** Relationship between plasma concentrations of nortriptyline (abscissa scale) and the prolongation of the PQ interval (ordinate scale) in old rats at 10 (●) and 60 (▲) min of nortriptyline infusion given at the rate 2.Q and 3.Q. Preinfusion PQ values were 61.6 ± 6 ms. The small number of animals prevented statistical analysis of these data. For comparison the two correlation lines obtained in young rats are shown.

Nortriptyline infusions (2.Q and 3.Q) induced marked alterations in the ECG tracing of old rats. The heart rate slowly increased in rats given the drug at infusion rate 2.Q while arrhythmias (extrasystoles, bigeminism, AV dissociation and ventricular tachycardia) were observed in rats treated at infusion rate 3.Q. An exception was rat No. 3 (Table 3) which was arrhythmic before the start of the experiment and did not show any worsening of the ECG tracing during nortriptyline infusion. In rat No. 4 arrhythmias appeared after about 10 min of infusion, at a plasma level of 6.75 µg/ml, and disappeared when plasma concentrations decreased during the second infusion. In rat Nos 5, 6 and 7, peak plasma levels ranged from 8.40 to 11.0 µg/ml; arrhythmias appeared between the 3rd and 5th min of infusion and the animals died from heart blockade within 10 minutes.

The electrical axis position was not altered at infusion rate 2.Q. At rate 3.Q the early appearance of arrhythmias masked any alteration of this parameter. The PQ interval increased in all the animals. When possible, this increase was related with corresponding plasma concentrations as shown in Figure 5, where the correlation lines calculated for young rats are provided for comparison. Although statistical analysis could not be carried out because of the limited number of observations, it seems likely that similar plasma concentrations of nortriptyline increase the PQ interval in young and old rats to much the same extent.

## Discussion

The cardiac effects of nortriptyline were studied in the rat by analyzing the relationship of drug plasma concentrations with three main effects: (1) changes in the heart rate; (2) deviation of the electrical axis and (3) prolongation of the PQ interval.

Plasma concentrations of nortriptyline used in the present experiments ranged from 0.15 µg/ml up to 11.0 µg/ml, thus covering both the therapeutic and the overdose limits found in humans (Asberg, Crönholm, Sjöqvist & Tuck, 1971; Asberg, 1974; Garattini & Morselli, 1975; Spiker *et al.*, 1975).

These concentrations were measured during nortriptyline infusion at 10 min (peak levels) and at 60 min (steady state levels). Heart determinations of nortriptyline, made at the same times, indicate that nortriptyline accumulation in cardiac tissue was greater by a factor of 12–23, thus showing behaviour rather similar to other tricyclic antidepressant drugs such as protriptyline and desipramine (Bianchetti *et al.*, 1976). Since this ratio was constant at any infusion rate, at any time and in both young and old animals, we assumed that plasma concentrations were an adequate parameter to relate with the cardiac effects induced by the drug. Nortriptyline, at plasma concentrations ranging from 0.15 to 6.0 µg/ml, mainly provoked an increase in heart rate, which was not plasma concentration-dependent, similar to the tachycardia induced in man by plasma levels ranging from 0.02 to 0.295 µg/ml (Freyschuss, Sjökvist, Tuck & Asberg, 1970). Only in a few rats, at the highest plasma concentrations, nortriptyline caused a feeble, inconsistent depressant effect on the heart rate.

Heart rate changes induced by tricyclic antidepressant drugs are supposed to result from the combination of cholinolytic, sympathomimetic and membrane stabilizing effects, (Cairncross & Gershon, 1962; Editorial, 1971; Elonen, Mattila & Saarnivaara, 1974; Barth & Muscholl, 1974). The prevalence of one effect over the others may be characteristic of each drug: nortriptyline, while sharing with desipramine and protriptyline the tachycardia effect, seems not to have the plasma concentration-related depressant effect on heart rate shown by the other compounds (Bianchetti *et al.*, 1977).

The two other cardiac parameters, rotation of the electrical axis and prolongation of the PQ interval, were correlated with plasma concentrations of nortriptyline ranging from 1.5 µg/ml to 6.0 µg/ml. Nortriptyline seems similar to desipramine but is more potent than protriptyline if we compare the threshold plasma concentrations producing 40° rotation of the electrical axis and 20% increase of the PQ interval.

These are 1.65 and 1.69 μg/ml for nortriptyline, 1.35 and 1.75 μg/ml for desipramine and 2.2 and 3.6 μg/ml for protriptyline (Bianchetti *et al.*, 1977). Apart from the threshold values, the correlations need further attention. The steeper relationship during the loading infusion (10 min) in comparison with that during the steady state (60 min) indicates that the cardiac alterations decrease with time of exposure to the drug, when the animal had been primed with relatively higher concentrations of nortriptyline. The mechanism of this decreasing sensitivity is not known but it recalls the tolerance that humans develop to some of the side effects of these drugs during chronic treatment (Alexanderson & Sjöqvist, 1971; Asberg, 1974; Burrows, Scoggins, Turececk & Davies, 1974).

Despite the very small number available, old rats gave useful information on the sensitivity of their heart to nortriptyline. The drug was much more cardiotoxic in old than in young rats, given equal doses. However, this difference disappeared when well defined pharmacological end points were related to similar plasma concentrations of nortriptyline in the two groups: arrhythmias occurred in old rats at plasma concentrations higher than 5.2 μg/ml, which was the threshold value for the onset of similar arrhythmias in young rats. Furthermore, the relationship between plasma levels and prolongation of the PQ interval was strikingly similar in both groups of animals. Although old rats showed signs of altered conduction (as indicated by the widened QRS interval) and increased vascular resistance (as suggested by the hypertrophy of the left ventricle), there was no evidence of their heart being supersensitive to nortriptyline. Slower drug metabolism and excretion, described in old animals for other classes of drugs (Kato & Takanaka, 1968; Klinger, 1969), is probably responsible for the higher plasma concentrations and therefore for the apparently higher toxicity of nortriptyline in old rats.

As already pointed out in the introduction, some authors have suggested that old people are more sensitive than young people to the cardiotoxic effects of antidepressant drugs. Since there is no information on the plasma levels of tricyclic antidepressant drugs in old patients, the more severe cardiotoxicity, might in part result from age-related alterations in drug metabolism and excretion.

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# RESPONSES OF MOTONEURONES TO ELECTROPHORETICALLY APPLIED DOPAMINE

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1 The effect of electrophoretically applied dopamine upon motoneurone excitability has been investigated. Field potentials originating from antidromically activated motoneurones were recorded from the ventral horn of the rat lumbar spinal cord.

2 Field potentials showed an increase in amplitude following electrophoretic application of dopamine. Dopamine was shown to be less potent than noradrenaline and 5-hydroxytryptamine in producing these changes.

3 Measurement of the transport number of dopamine suggests that the relatively low potency of dopamine cannot be attributed to differences in ionic mobilities between the amines.

4 Electrophoretic application of $\alpha$-flupenthixol was shown to discriminate between dopamine and 5-hydroxytryptamine responses. Dopamine responses were profoundly reduced.

5 Electrophoretically applied $\alpha$-flupenthixol also discriminated between dopamine and noradrenaline. Noradrenaline responses were consistently potentiated by $\alpha$-flupenthixol. The possibility is discussed that dopamine may not merely be a precursor for noradrenaline in the rat spinal cord.

## Introduction

Using the Falck-Hillarp technique, Dahlström & Fuxe (1965) reported the existence of descending monoaminergic pathways in the spinal cord. They observed noradrenaline and 5-hydroxytryptamine containing terminals in close proximity to lumbar motoneurones. Subsequent experiments have provided good evidence to suggest a neurotransmitter role for the amines in the spinal cord. In contrast, the relatively small quantities of dopamine present in the spinal cord have been regarded as having an entirely precursor role to noradrenaline. Recently, however, Commissiong & Sedgewick (1974) and Magnusson (1973) have suggested that dopamine is present in the spinal cord in amounts which indicate that the amine may have a neurotransmitter role.

The present study was carried out to determine whether dopamine applied locally by microelectrophoresis has any pharmacological effects on spinal motoneurones and whether these effects can be blocked by a drug considered to be a specific antagonist of responses to dopamine.

## Methods

Experiments were performed on Wistar rats of either sex anaesthetized with fluothane. Blood pressure and ECG were routinely monitored. Laminectomy was performed between lumbar and sacral segments and ventral roots were cut about 1 cm from the cord.

Drugs were applied by microelectrophoresis from five-barrelled micropipettes. Solutions were made up as follows: 5-hydroxytryptamine bimaleate (Koch-Light) 0.2 M, pH 3.0, noradrenaline hydrochloride (Koch-Light) 0.2 M, pH 3.5, the diacetate salt of $\alpha$-flupenthixol (Lundbeck) 0.2 M, pH 3.5 and dopamine hydrochloride (Sigma) 0.2 M, pH 3.5.

Extracellular field potential responses of lumbar motoneurones were evoked by regular antidromic stimulation of the central end of transected ventral roots and recorded through a sodium chloride filled barrel of the micropipette. Square wave pulses of 0.5 ms duration of varying intensity were used to deliver stimulation to ventral roots. Field potentials originating from motoneurones were displayed on an oscilloscope and their amplitude recorded on a pen writing polygraph. Field potentials changed in amplitude when drugs were applied. The change of the field potential amplitude and the period for which the change persisted was defined as the response to the electrophoretically applied drug. Quantification of drug effects involved measuring the area enclosed by the response. Response areas were measured with a planimeter. An example of the procedure is shown in Figure 1d (see also legend).

Further details of the experimental procedures have been published elsewhere (Barasi & Roberts, 1974).

*Determination of transport numbers*

A series of *in vitro* experiments was performed to investigate the transport number of dopamine and noradrenaline. Transport numbers were obtained by the use of five-barrelled micropipettes similar to those used in the *in vivo* experiments.

Freeze-dried (±)-noradrenaline [carbinol-$^{14}$C]-(±)-bitartrate (specific activity 35 mCi/mmol) was mixed with non-radioactive noradrenaline to provide a final solution of 0.2 M. A solution of similar molarity was made from [ethylamine-2-$^{14}$C]-dopamine hydrochloride and non-radioactive dopamine hydrochloride. The pH of the final solutions was 3.5.

The effects of 25, 50, 100 and 150 nA ejecting current was studied in all pipettes. On each occasion the pipette tip was lowered into a perspex vial containing 1 ml of 0.165 M NaCl. An electrophoretic current was applied for 10 min following which the 1 ml sample was transferred to a counting vial containing 10 ml of scintillator (Nuclear Enterprises NE 260). This was repeated three times for each current. Disintegrations originating from each radioactive sample were counted during a 10 min period in a Nuclear Enterprises liquid scintillation counter.

## Results

*Motoneurone field potentials*

The amplitude of antidromically evoked motoneurone field potentials depended upon the position of the micropipette and the intensity of the ventral root stimulation. Field potentials were generally first recorded at a depth of about 1000 μm from the dorsal surface of the cord. As the micropipette penetrated further towards the ventral horn the amplitude of the recorded potential increased frequently to about 0.7 mV. Increasing the intensity of ventral root stimulation (to a maximum of about 0.3 V) brought about a concomitant increase in field potential amplitude. Stimulation voltage was adjusted at a level twice that required to give a just maximum field potential. Figure 1a illustrates a typical antidromically evoked field potential recorded from the rat spinal cord.

Although no obvious relationship was noted between responsiveness of the field potential to locally applied drugs and location (depth) of the micropipette, we did find that the larger field potentials recorded between about 1500 and 2000 μm were influenced less by drugs.

Providing the blood pressure remained constant, field potential amplitude fluctuated little. However, almost invariably, a fall in blood pressure resulted in an increase in field potential amplitude and some preparations were abandoned for this reason.

*Response to electrophoretically applied dopamine*

Dopamine was applied electrophoretically whilst recording antidromically evoked motoneurone field potentials in 91 positions in 21 animals. Currents used to eject dopamine varied between 75 and 150 nA.

An example of the increase in motoneurone field potential in response to the application of dopamine is shown in Figure 1b. The increase in field potential amplitude was dependent upon the intensity of the electrophoretic current and duration of the application. The extent of the increase varied between about 30% and 100%. On 45 occasions (50%) the field potential was increased in amplitude whilst on only 2 occasions was a small reduction in amplitude recorded. Dopamine was ineffective on 45 occasions (50%) in changing field potential amplitude. In 18 of these studies 5-hydroxytryptamine or noradrenaline evoked marked changes in the field potential.

Figure 1c and d shows how the response to the same amine varied from one recording position to another. The more usual response was a graded increase in amplitude (Figure 1d), the amplitude of the field potential showed an approximately regular increase during the application of the amine. In some recording positions discrete step-like increases in amplitude were recorded. Figure 1c illustrates a study in which this effect was observed, and it can be seen that both 5-hydroxytryptamine and dopamine increased the field potential amplitude incrementally. In those positions in the spinal cord where noradrenaline and dopamine were effective and were ejected with similar currents for similar periods of time, noradrenaline invariably produced the larger response. In 10 such studies the average noradrenaline response was 2.2 ± 0.2 times greater in area than the dopamine response.

*Antagonism studies*

*Discrimination betwen dopamine and 5-hydroxytryptamine.* 5-Hydroxytryptamine and dopamine were ejected with electrophoretic currents whose intensity and duration were adjusted to give comparable response areas; 25–50 nA of α-flupenthixol was then applied electrophoretically for 10–15 minutes. The results of one study is shown in Figure 2. Each filled symbol on the graph (Figure 2c) represents the area enclosed by a single response to 5-hydroxytryptamine or dopamine. Two excerpts from the record are shown. Figure 2a illustrates responses to 5-hydroxytryptamine and dopamine before the application of α-flupenthixol whilst Figure 2b illustrates the size of the amine responses following application of the antagonist. The open circles on the graph show that the amplitude of the individual field potentials measured just before application of either agonist was uninfluenced by α-flupenthixol. The antagonist did, however, reduce dopamine responses to 25% of pre-

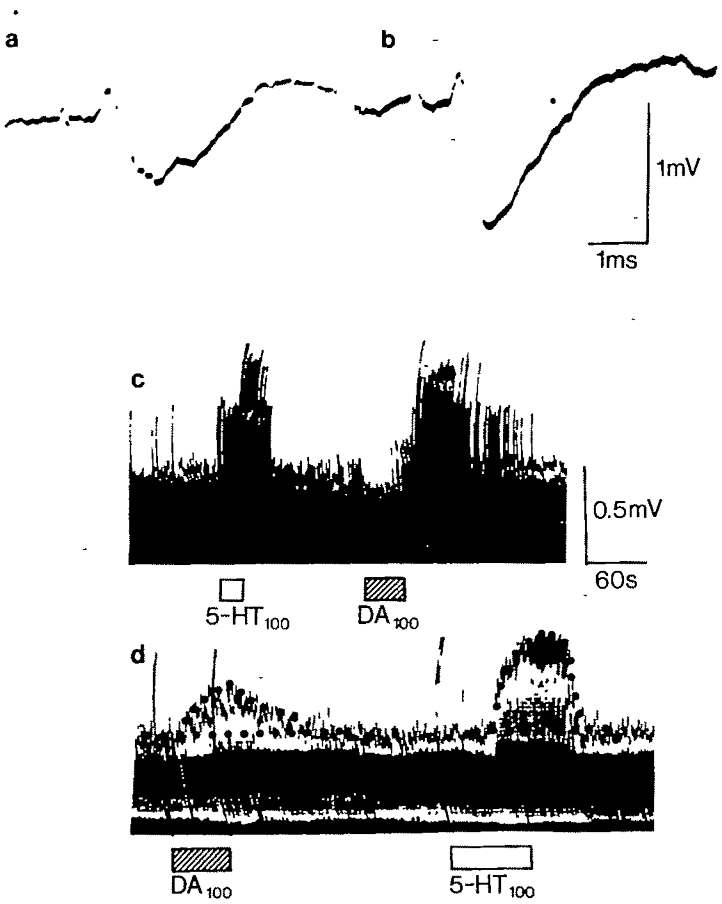

**Figure 1** Responses of motoneurone field potentials to electrophoretically applied amines. (a) The negative-going motoneurone field potential recorded from the ventral horn in response to antidromic stimulation of ventral roots. Field potentials were evoked at the rate of between 12 and 20 per minute. (b) Increase in amplitude of the field potential in response to the electrophoretic application of 100 nA of dopamine (DA). (c) Step-like increase in field potential amplitude in response to electrophoretically applied amine. Each line represents one motoneurone field potential. Changes in the height of the lines reflect changes in amplitude of the field potential. Bars and numbers beneath the record indicate the duration and intensity (nA) (respectively) of electrophoretic currents (d) Graded response to electrophoretically applied amines. Responses were quantified by measuring the area enclosed by the dotted line.

antagonist levels without affecting 5-hydroxytryptamine responses.

Recovery from the maximal dopamine blockade began shortly after termination of the α-flupenthixol application but was incomplete 110 min later. α-Flupenthixol has shown selective blockade of this nature on four occasions. In 4 other studies, however, the antagonist blocked responses to both 5-hydroxytryptamine and dopamine, although on no occasion was the 5-hydroxytryptamine response preferentially blocked. High doses of α-flupenthixol (>75 nA for 5 min or more) decreased the amplitude of the motoneurone field potential.

*Discrimination between dopamine and noradrenaline.* Electrophoretically applied dopamine has a relatively weak effect on motoneurone field potentials, and obtaining alternate reproducible dopamine and noradrenaline responses proved rather difficult. Figure 3c shows a study in which α-flupenthixol successfully discriminated between the two amines. Following two control applications of each amine, 50 nA of α-flupenthixol was applied for 7 minutes. The dopamine response was reduced to 30% of pre-antagonist level but showed complete recovery. The effect of α-flupenthixol on noradrenaline responses was quite different: there was

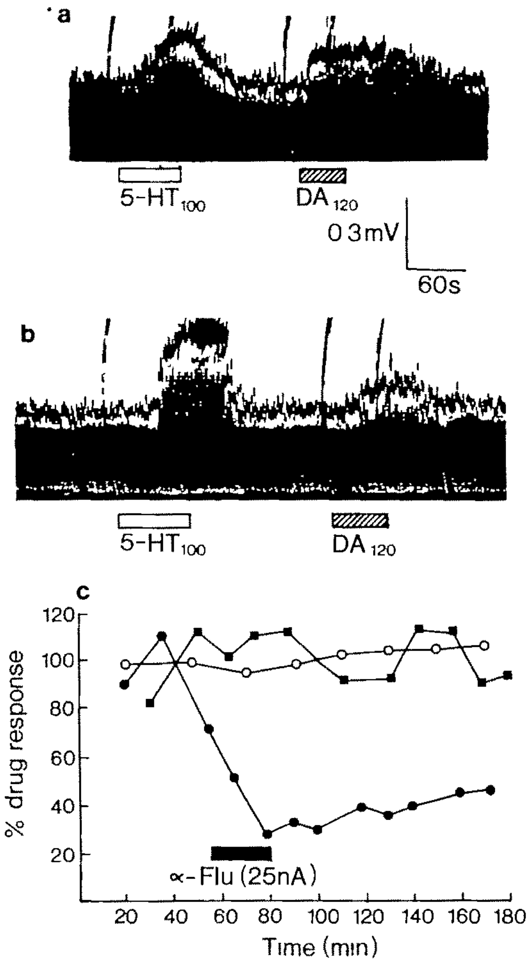

**Figure 2** Effect of $\alpha$-flupenthixol on 5-hydroxytryptamine and dopamine responses. (a) Excerpts from the records illustrating the responses of motoneurone field potentials to 5-hydroxytryptamine (5-HT) and dopamine (DA) (b) Responses to 5-hydroxytryptamine and dopamine following the application of $\alpha$-flupenthixol The amplitude and time calibration marks are applicable to both excerpts. (c) Graph representing the entire study of which (a) and (b) were excerpts. $\alpha$-Flupenthixol ($\alpha$-Flu) has discriminated between the two amine responses. The 100% response level corresponds to the mean amine response before application of $\alpha$-flupenthixol. 5-Hydroxytryptamine responses (■); dopamine responses (●), individual field potential amplitude evoked in the absence of locally applied amine (O)

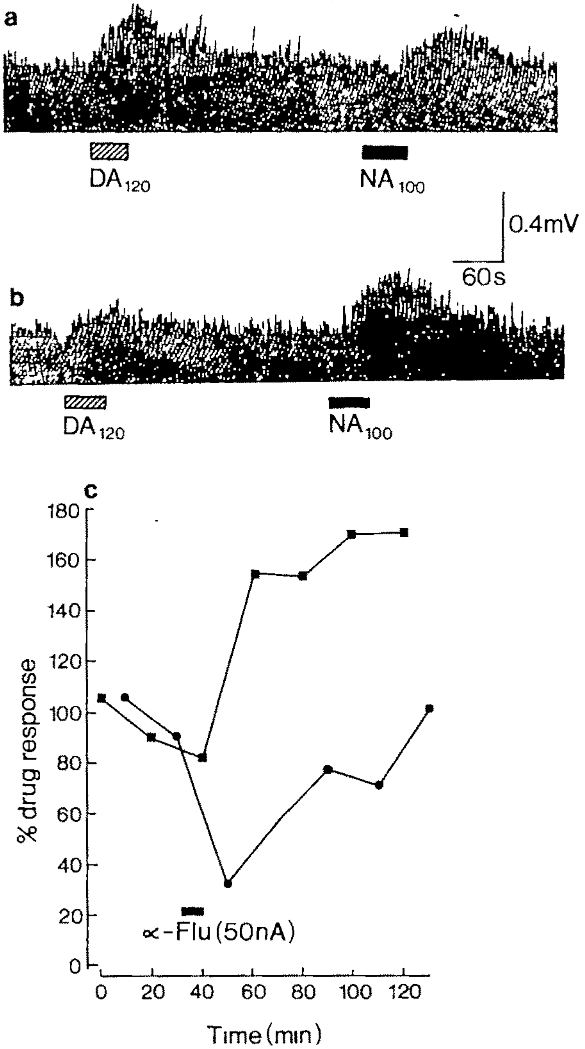

**Figure 3** Effect of $\alpha$-flupenthixol on dopamine and noradrenaline responses. (a) Excerpts from records illustrating responses to dopamine (DA) and noradrenaline (NA) in the absence of antagonist. Calibration marks refer to both (a) and (b). (b) Responses to dopamine and noradrenaline following the application of $\alpha$-flupenthixol (c) Graph illustrating the complete study. The dopamine antagonist has reduced the dopamine response and potentiated the noradrenaline response. The 100% response level corresponds to the mean amine response before application of $\alpha$-flupenthixol ($\alpha$-Flu). Noradrenaline responses (■); dopamine responses (●).

a clear potentiation which in this study showed no recovery. Figure 3a and b illustrates selected responses from the study.

Potentiation of the noradrenaline response following $\alpha$-flupenthixol was observed on 4 occasions and the antagonist discriminated between the 2 amines in 5 studies.

In several studies spontaneous firing was recorded following application of the antagonist. The firing was usually associated with a reduction in the control field potential amplitude, that is on those occasions when excess antagonist was applied.

### *Transport number determinations*

The average transport number obtained from 4 micropipettes for noradrenaline was 0.155 ± 0.002 (mean ± s.e. mean) and from 5 micropipettes for dopamine was 0.392 ± 0.001. These values represent the average of 15 release readings from each pipette. The resting release (corresponding to 0 nA) was subtracted from the ejection release before the transport number was calculated. The mean resting release from all pipettes was 1.3 ± 0.2 pmol/min per barrel.

## **Discussion**

The increase in field potential following dopamine application probably reflects an increase in the excitability of motoneurones (Barasi & Roberts, 1974). The ability of $\alpha$-flupenthixol to discriminate between responses to 5-hydroxytryptamine and dopamine and noradrenaline and dopamine suggests that dopamine responses do not result from an action upon a generalized amine receptor.

The specificity of monoamine receptor blocking drugs varies markedly. However, it seems likely that the thioxanthene derivative $\alpha$-flupenthixol is a relatively specific dopamine antagonist. A comparison of the relative potencies of a range of antipsychotic drugs in inhibiting dopamine-stimulated adenylate cyclase (Iversen, 1975), suggests that $\alpha$-flupenthixol is the most potent of the drugs currently available.

Two studies have been described (Ben-Ari & Kelly, 1974; House & Ginsborg, 1976) which indicate that dopamine effects can be blocked by $\alpha$-flupenthixol. The present experiments compare the effect of $\alpha$-flupenthixol on responses to dopamine, noradrenaline and 5-hydroxytryptamine. The results suggest that spinal motoneurones or adjacent units are sensitive to locally applied dopamine and that the receptors involved are blocked by an antagonist believed to be specific for dopamine.

$\alpha$-Flupenthixol potentiated responses of motoneurones to noradrenaline. This unexpected observation seems to support the suggestion of Brown & Makman (1972) and Kebabian, Petzgold & Greengard (1972) that dopamine receptors are pharmacologically distinct from $\beta$-adrenoceptors. The potentiation of noradrenaline responses by $\alpha$-flupenthixol has not been reported previously and no explanation of this effect is presently justified.

Values for the transport number of noradrenaline from bitartrate solutions are comparable to previous determinations (Bradshaw, Roberts & Szabadi, 1973; Candy, Boakes, Key & Worton, 1974). However, the transport number for dopamine from hydrochloride solutions was found to be more than twice that recorded for noradrenaline. Consequently the differences in the size of the noradrenaline and dopamine responses cannot be attributed to differences in mobilities of the two ions and suggests that dopamine is much less potent on lumbar motoneurones than noradrenaline.

It has been suggested (Candy *et al.*, 1974) that electrophoretically applied drugs may, under diffusional influences, travel many hundreds of microns from the micropipette tip. Under these circumstances, it is possible that the present effects may have been mediated indirectly via interneurones close to the motoneurones. However, it was only rarely that recordings of spontaneous activity ever accompanied the motoneurone field potential. It thus seems unlikely that the effects of electrophoretically applied amine were consistently being mediated via interneurones.

The difference in response characteristics between one recording site and another may be associated with the spatial relationship between the recording electrode and the motoneurone. The step-wise increase in field-potential amplitude illustrated in Figure 1c may reflect the lowering of the threshold of two motoneurones located close to the recording microelectrode. In the absence of amine the motoneurones would remain univaded by the antidromic action potential. Following the application of amine the threshold of the motoneurones was perhaps lowered and the antidromic impulse invaded the cells. The more regular shape of drug responses illustrated in Figure 1d probably results from similar changes in excitability occurring in motoneurones at some distance from the recording microelectrode.

There is conflicting evidence concerning the existence of dopamine within the spinal cord. Magnusson & Rosengren (1963), Atack (1963), Anton & Sayer (1964) and Laverty & Sharman (1965), all reported dopamine concentrations within the range 10–20 ng/gram. McGeer & McGeer (1962), however, found dopamine in concentrations of about 1000 ng/g in rabbit and rat cord. More recently Commissiong & Sedgewick (1974), initially investigating dopamine concentrations in the spinal cord of rat, reported concentrations of 130 ng/gram. Subsequently in human spinal cord the same authors found similar (up to 190 ng/g) concentrations (Commissiong & Sedgewick, 1975).

Magnusson (1973) monitored changes in concentration of noradrenaline and dopamine in rat spinal cord following transection and noted a different time course for the disappearance of the two amines. This evidence may suggest that the dopamine existing in the spinal cord is not inevitably associated with noradrenaline.

Further evidence supporting the existence of a descending dopamine pathway comes from experiments in which electrical stimulation of the substantia nigra changed spinal monosynaptic reflex activity (York, 1972). These effects were not mediated via the supraspinal structures but were consistently blocked by agents thought to have dopamine blocking activity (chlorpromazine and haloperidol).

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# INHIBITION BY $Cd^{2+}$, VERAPAMIL AND PAPAVERINE OF $Ca^{2+}$-INDUCED CONTRACTIONS IN ISOLATED CEREBRAL AND PERIPHERAL ARTERIES OF THE DOG

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1 In helically cut strips of canine cerebral arteries exposed to $Ca^{2+}$-free media and depolarized by $K^+$, the addition of $Ca^{2+}$ caused biphasic (transient and sustained) contractions, while in coronary and mesenteric arteries, the addition of $Ca^{2+}$ produced a sustained contraction sometimes preceded by a slight transient contraction.

2 These $Ca^{2+}$-induced contractions were attenuated by $Cd^{2+}$ (5 to 100 μM) in a dose-dependent manner, the attenuation being greater in cerebral than in coronary and mesenteric arteries. The inhibitory effect of $Cd^{2+}$ was prevented and partially reversed by 1 mM cysteine.

3 Verapamil and papaverine were also effective in attenuating the $Ca^{2+}$-induced contractions in cerebral and peripheral arteries: susceptibility to verapamil was in the order, cerebral > coronary > mesenteric, while that to papaverine was in the order, cerebral = coronary > mesenteric.

4 It may be concluded that the agents that interfere with trans-membrane influxes of $Ca^{2+}$ cause a greater relaxation in cerebral than in peripheral arteries, as is seen with papaverine, a non-specific vasodilator.

## Introduction

It has already been shown that cerebral arterial smooth muscles both from man and animals respond differently from peripheral arterial smooth muscles when vasoconstrictor and vasodilator agents are applied (Bohr, Goulet & Taquini, 1961; Uchida, Bohr & Hoobler, 1967; Toda & Fujita, 1973; Toda, 1974a; Dalske, Harakal, Sery & Menkowitz, 1974; Müller-Schweinitzer, 1976). Our previous data showed that a triphasic pattern of responses, a rapid contraction, rapid relaxation, and sustained contraction, is induced by the addition of $Ca^{2+}$ in isolated cerebral arteries of the dog after a long exposure to $Ca^{2+}$-free media including excess $K^+$ (Toda, 1974b). This pattern of responses clearly differs from that observed in peripheral arteries.

Cadmium ions and verapamil interfere with the influx of $Ca^{2+}$ across cell membranes in cardiac and vascular smooth muscles (Fleckenstein, Tritthard, Fleckenstein, Herbst & Gruen, 1969; Kaufmann, Tritthart, Rost & Fleckenstein, 1970; Toda, 1973a; 1976a), resulting in vasodilatation. On the other hand, papaverine causes a relaxation of smooth muscles by mechanisms relating not only to influxes of $Ca^{2+}$ but also to intracellular $Ca^{2+}$ sequestration (Carpenedo, Toson, Furlanut & Ferrari, 1970; Tashiro & Tomita, 1970) and oxidative phosphorylation (Santi, Ferrari & Contessa, 1964; Ferrari & Carpenedo, 1968).

The present study was undertaken to determine the inhibitory effect of $Cd^{2+}$, verapamil and papaverine on arterial contractions induced by $Ca^{2+}$ and to evaluate the susceptibility of canine cerebral and peripheral arteries to vasodilator agents acting directly on vascular smooth muscle cells.

## Methods

Mongrel dogs of either sex, weighing between 7 to 16 kg, were anaesthetized with intraperitoneal injections of sodium pentobarbitone in a dose of 50 mg/kg and were killed by bleeding from the common carotid arteries. The brain and heart were rapidly removed, and the basilar and middle cerebral arteries (0.6 to 0.9 mm outside diameter) and the ventral interventricular branch of left coronary arteries (0.6 to 0.9 mm) were isolated. The distal portion of the superior mesenteric arteries (0.6 to 1.0 mm) was also isolated. The basilar and middle cerebral arteries responded to vasoactive agents in the same way; therefore, the term 'cerebral arteries' in this paper includes both arteries. The specimen was cut helically into strips approximately 20 mm long. These strips were fixed vertically between hooks under a resting tension of 1.5 g in a muscle bath of 20 ml capacity, containing the nutrient solution. Hooks anchoring the upper end of the strip were connected to the lever of a force-displacement transducer (Nihonkoden Kogyo Co., Tokyo, Japan). The bathing fluid was aerated with a mixture of 95% $O_2$ and 5% $CO_2$ and maintained at 37 ± 0.5°C. The composition of the solution was as follows (mM): $Na^+$ 162.1, $K^+$ 5.4, $Ca^{2+}$ 2.2, $Cl^-$ 157.0, $HCO_3^-$ 14.9, and glucose 5.6. The pH of the solutions was 7.2 to 7.3. In order to raise external concentrations of $K^+$, the KCl solution was added directly to the bathing media. Osmotic adjustment was not made when external $K^+$ was raised and external $Ca^{2+}$ was removed. The preparations were allowed to equilibrate for 90 to 120 min in control media and during the equilibration period, the bathing media were replaced every 15 to 20 minutes.

Isometric contractions of arterial strips were displayed on an ink-writing oscillograph (Sanei Sokki Co., Tokyo, Japan). The contractile response to 25 mM $K^+$ was first obtained and the preparations were washed three times with normal fluid. After an equilibration period of 35 to 45 min, preparations were exposed for 60 min to $Ca^{2+}$-free media, during which time the solution was replaced twice every 20 min, and $K^+$ (25 mM) was added. After the $K^+$-induced contraction levelled off, $Ca^{2+}$ in a concentration of 2.2 mM was added. Some preparations were treated for 20 min with blocking agents, before the addition of $K^+$. Contractions induced by $Ca^{2+}$ relative to those by $K^+$ (25 mM) in control media in the same preparations were calculated. The values obtained in the presence and absence of treatment with blocking agents were compared. Results shown in the text, tables and figures represent mean values ± s.e. means. Statistical analyses in paired preparations were made using Student's *t* test.

Drugs used were verapamil hydrochloride (Eisai Pharmaceutical Co., Tokyo, Japan), papaverine hydrochloride, L-cysteine hydrochloride and glutathione.

## Results

### *Comparisons of the response to $Ca^{2+}$ in cerebral, coronary and mesenteric arteries*

In helically cut strips of cerebral arteries exposed for 60 min to $Ca^{2+}$-free media and depolarized by 25 mM $K^+$, the addition of 2.2 mM $Ca^{2+}$ caused a phasic contraction followed by sustained contraction (Figure 1). Such a phasic contraction was also elicited by $Ca^{2+}$ in $Ca^{2+}$-free media in the absence of the treatment with 25 mM $K^+$ (Table 1). In coronary and mesenteric arterial strips exposed to $Ca^{2+}$-free media and treated with 25 mM $K^+$, the addition of $Ca^{2+}$ caused a sustained contraction which was sometimes preceded by a slight, phasic contraction (Figure 1).

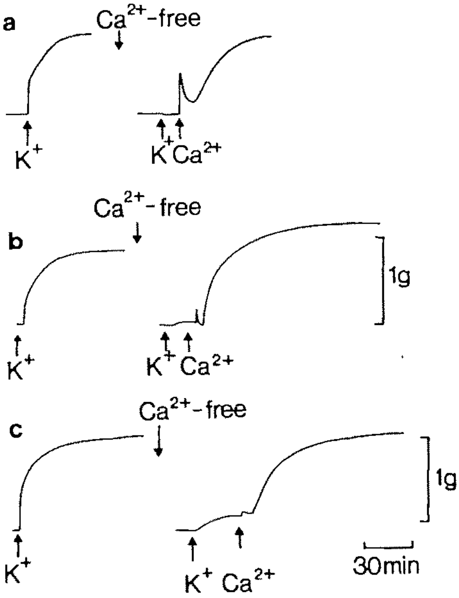

**Figure 1** Comparison of the effect of $K^+$ and $Ca^{2+}$ on (a) basilar (b) coronary and (c) mesenteric arteries isolated from the same dog. The response to $K^+$ (25 mM) was first obtained in control media Preparations were then exposed for 60 min to $Ca^{2+}$-free media, and $K^+$ (25 mM) and $Ca^{2+}$ (2.2 mM) were added successively.

Contractile responses of these arteries to $K^+$ and $Ca^{2+}$ in $Ca^{2+}$ (2.2 mM)-containing and $Ca^{2+}$-free media are summarized in Figure 2. Contractions induced by $K^+$ in $Ca^{2+}$-free media were appreciably less in cerebral than in coronary and mesenteric arteries, and phasic contractions by $Ca^{2+}$, shown as 'A' on the abscissa scale in Figure 2, were significantly greater.

### *Inhibition by $Cd^{2+}$ of the $Ca^{2+}$-induced contraction*

Treatment with $Cd^{2+}$ (5 to 100 μM) caused a slight, persistent relaxation. This relaxation was not dependent upon concentrations of $Cd^{2+}$, because the preparations relaxed almost completely in control media, therefore relaxation induced by vasodilator agents was always slight and inconsistent from preparation to preparation.

The contractile response of cerebral arteries to $Ca^{2+}$ was reduced by treatment for 20 min with $Cd^{2+}$ (5 to 100 μM) in a dose-dependent manner (Table 1). Both the phasic and sustained contractions were attenuated,

**Table 1** Inhibition by Cd$^{++}$ of the Ca$^{++}$-induced contraction in isolated cerebral, coronary and mesenteric arteries

| | | | | | | *Responses (mg) to* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *Ca$^{2+}$ 2.2 mM* | | | | |
| *Arteries and conditions* | n | *K$^{+}$ 25 mM* | *Ca$^{2+}$ removal* | *Cd$^{2+}$* | *K$^{+}$ 25 mM* | *A* | *B* | *C* | *Cysteine 1 mM* | *Cysteine + Ca$^{2+}$* |
| *Cerebral* | | | | | | | | | | |
| Control | 23 | 838±70 | −110±27 | | 24±9 | 436±66<br>(53±4.7%) | 160±26 | 880±69<br>(110±5.1%) | −378±101 | 505±62<br>(61±4.9%) |
| Control* | 8 | 806±118 | −68±30 | | | 416±16<br>(51±5.3%) | 246±96 | | | |
| Cd$^{2+}$ 5 µM | 13 | 896±128 | −180±54 | −48±17 | 26±9 | 350±59<br>(39±6.9%) | 18±3 | 576±95<br>(67±6 9%)** | 466±91<br>(52±12%) | 920±116<br>(108±5.1%) |
| Cd$^{2+}$ 20 µM | 17 | 720±67 | −144±43 | −36±12 | 60±19 | 68±29<br>(7.6±3.2%)** | 19±9 | 132±38<br>(19±5 1%)** | 272±76<br>(39±9 0%) | 420±76<br>(59±8.3%) |
| Cd$^{2+}$ 100 µM | 6 | 787±147 | −146±65 | −74±19 | 0 | 0<br>(0%)** | 0 | 0<br>(0%)** | 103±58<br>(14±5.9%) | 103±58<br>(14±5.9%) |
| *Coronary* | | | | | | | | | | |
| Control | 26 | 930±72 | −32±26 | | 48±14 | | | 1136±78<br>(117±4 9%) | −330±62 | 809±93<br>(88±5.3%) |
| Cd$^{2+}$ 5 µM | 9 | 910±70 | −58±18 | −6±3 | 3±2 | | | 1010±102<br>(110±6.6%) | −318±49 | 705±68<br>(78±7.6%) |
| Cd$^{2+}$ 20 µM | 10 | 1236±115 | −202±82 | −118±44 | 0 | | | 504±112<br>(40±7.2%)** | 306±57<br>(27±5.7%) | 810±102<br>(67±6.2%) |
| Cd$^{2+}$ 100 µM | 5 | 1502±297 | −342±143 | −50±15 | 4±2 | | | 2±2<br>(0%)** | 660±165<br>(49±10%) | 661±165<br>(49±10%) |
| *Mesenteric* | | | | | | | | | | |
| Control | 27 | 953±99 | −42±22 | | 163±28 | | | 918±95<br>(98±5.4%) | −195±81 | 725±93<br>(77±6.2%) |
| Cd$^{2+}$ 5 µM | 9 | 1192±176 | −56±23 | −9±3 | 78±66 | | | 1053±195<br>(87±7.2%) | 210±90<br>(20±10%) | 1241±141<br>(113±11%) |
| Cd$^{2+}$ 20 µM | 8 | 1860±212 | −120±50 | −76±52 | 52±16 | | | 1097±233<br>(59±11%)* | 544±122<br>(28±5.9%) | 1641±220<br>(87±8.0%) |
| Cd$^{2+}$ 100 µM | 7 | 1668±202 | −50±41 | −66±18 | 90±23 | | | 37±22<br>(2.0±1.3%)** | 1002±113<br>(67±10%) | 1058±127<br>(68±10%) |

Control* in cerebral arteries; Contractions induced by Ca$^{2+}$ were obtained in Ca$^{2+}$-free media without the addition of K$^{+}$ (values obtained in the previous study, Toda, 1974b). A, B, C: see the legend for Figure 2. Numbers in parentheses indicate the contractions relative to those induced by 25 mM K$^{+}$ in control media. Minus indicates relaxation.

Significantly different from respective controls, **$P<0.001$, *$P<0.01$.

**Table 2** Inhibition by verapamil of the $Ca^{2+}$-induced contraction in isolated cerebral, coronary and mesenteric arteries of the dog

| | | | | | | Response (mg) to | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | $Ca^{2+}$ 2.2 mM | | | | |
| *Arteries and conditions* | n | $K^+$ 25 mM | $Ca^{2+}$-removal | Verapamil | $K^+$ 25 mM | A | B | C | + $Ca^{2+}$ 4.4 mM | $Ca^{2+}$ (total) |
| *Cerebral* | | | | | | | | | | |
| Control | 23 | 838±70 | −110±27 | | 24±9 | 436±66<br>(53±4.7%) | 160±26 | 880±69<br>(110±5·1%) | −104±20 | 771±61<br>(92±5.2%) |
| Verapamil 50 nM | 10 | 1348±117 | −184±90 | −86±21 | 12±7 | 282±82<br>(20±5.5%)** | 226±88 | 650±83<br>(49±4.1%)** | 8±32 | 658±78<br>(50±5.0%) |
| Verapamil 0.2 μM | 7 | 1054±228 | −140±43 | −52±16 | 10±14 | 90±68<br>(8.6±2.1%)** | 70±52 | 212±51<br>(23±4.8%)** | 128±29 | 340±73<br>(37±5.9%) |
| Verapamil 1 μM | 4 | 1176±47 | −46±22 | −40±21 | 5±2 | 0 | 0 | 46±13<br>(3.5±0.7%)** | 116±65 | 160±70<br>(14±5.9%) |
| *Coronary* | | | | | | | | | | |
| Control | 26 | 930±72 | −32±26 | | 48±14 | | | 1136±78<br>(117±4.9%) | −120±70 | 1019±72<br>(108±4.5%) |
| Verapamil 50 nM | 7 | 1394±190 | −46±15 | −24±4 | 0±56 | | | 1104±159<br>(82±10%)* | 58±72 | 1162±170<br>(85±7.8%) |
| Verapamil 0.2 μM | 8 | 1216±243 | −40±31 | −18±8 | 4±6 | | | 406±109<br>(32±5.5%)** | 250±35 | 658±119<br>(59±5.9%) |
| Verapamil 1 μM | 5 | 980±218 | −30±10 | −5±3 | 6±4 | | | 32±18<br>(2.4±1.3%)** | 88±28 | 120±44<br>(14±3.5%) |
| *Mesenteric* | | | | | | | | | | |
| Control | 27 | 953±99 | −42±22 | | 163±28 | | | 918±95<br>(98±5.4%) | −296±109 | 625±95<br>(66±4.7%) |
| Verapamil 50 nM | 7 | 1014±187 | −38±3 | −10±4 | 34±16 | | | 834±208<br>(80±7.8%) | −116±46 | 720±241<br>(68±10%) |
| Verapamil 0.2 μM | 8 | 1432±274 | −18±7 | −14±4 | 36±13 | | | 686±199<br>(42±6.2%)** | 86±73 | 773±206<br>(49±4.8%) |
| Verapamil 1 μM | 5 | 942±106 | −33±29 | −7±3 | 16±8 | | | 127±65<br>(14±8.1%)** | 144±47 | 271±108<br>(31±14%) |

A, B, C: see the legend for Figure 2. Numbers in parentheses indicate the contractions relative to those induced by 25 mM $K^+$ in control media. Minus indicates relaxation.
Significantly different from respective controls, $^{**}P<0.001$; $^{*}P<0.01$.

**Table 3** Inhibition by papaverine of the $Ca^{2+}$-induced contraction in isolated cerebral, coronary and mesenteric arteries of the dog.

| | | Response (mg) to | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | $Ca^{2+}$ 2.2 mM | | |
| *Arteries and conditions* | n | *$K^+$ 25 mM* | *$Ca^{2+}$-removal* | *Papaverine* | *$K^+$ 25 mM* | *A* | *B* | *C* |
| *Cerebral* | | | | | | | | |
| Control | 23 | 838 ± 70 | −110 ± 27 | | 24 ± 9 | 436 ± 66<br>(53 ± 4.7%) | 160 ± 26 | 880 ± 69<br>(110 ± 5.1%) |
| Papaverine 1 μM | 8 | 608 ± 88 | −60 ± 24 | −24 ± 4 | 66 ± 36 | 300 ± 128<br>(42 ± 11%) | 118 ± 84 | 528 ± 86<br>(86 ± 4.3%)† |
| Papaverine 5 μM | 11 | 822 ± 122 | −126 ± 41 | −76 ± 23 | 29 ± 14 | 162 ± 62<br>(16 ± 4.4%)** | 57 ± 8 | 360 ± 54<br>(44 ± 2.1%)** |
| Papaverine 20 μM | 5 | 1122 ± 216 | −144 ± 47 | −82 ± 36 | 12 ± 6 | 150 ± 46<br>(12 ± 2 4%)** | 14 ± 4 | 358 ± 62<br>(31 ± 4.7%)** |
| *Coronary* | | | | | | | | |
| Control | 26 | 930 ± 72 | −32 ± 26 | | 48 ± 14 | | | 1136 ± 78<br>(117 ± 4.9%) |
| Papaverine 1 μM | 7 | 1090 ± 114 | −60 ± 24 | −68 ± 14 | 86 ± 36 | | | 982 ± 182<br>(88 ± 9.1%)† |
| Papaverine 5 μM | 23 | 1104 ± 72 | −132 ± 45 | −148 ± 37 | 46 ± 13 | | | 622 ± 110<br>(56 ± 4.9%)** |
| Papaverine 20 μM | 11 | 1304 ± 198 | −102 ± 38 | −148 ± 24 | 5 ± 2 | | | 502 ± 129<br>(38 ± 4.9%)** |
| *Mesenteric* | | | | | | | | |
| Control | 27 | 953 ± 99 | −40 ± 22 | | 163 ± 28 | | | 918 ± 95<br>(98 ± 5.4%) |
| Papaverine 1 μM | 4 | 1138 ± 284 | −65 ± 28 | −15 ± 9 | 163 ± 77 | | | 1156 ± 332<br>(98 ± 3 6%) |
| Papaverine 5 μM | 15 | 1374 ± 126 | −44 ± 19 | −38 ± 9 | 150 ± 40 | | | 958 ± 113<br>(71 ± 6.8%)* |
| Papaverine 20 μM | 12 | 1390 ± 113 | −50 ± 20 | −82 ± 26 | 76 ± 34 | | | 562 ± 93<br>(40 ± 6.0%)** |

A, B, C see the legend for Figure 2. Numbers in parentheses indicate the contractions relative to those induced by 25 mM $K^+$ in control media. Minus indicates relaxation.
Significantly different from respective controls, $^{**}P<0.001$; $^{*}P<0.01$; $^{\dagger}P<0\ 02$.

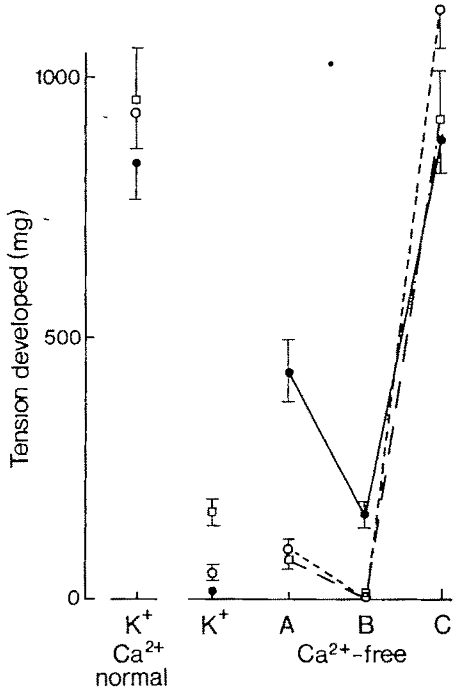

**Figure 2** Contractile responses to K⁺ and $Ca^{2+}$ of cerebral (●), coronary (○) and mesenteric (□) arteries exposed to control and $Ca^{2+}$-free media. Concentration of K⁺.25 mM. A, initial contraction induced by 2.2 mM $Ca^{2+}$; B, level of the minimum tension developed following the addition of $Ca^{2+}$; C, sustained contraction induced by $Ca^{2+}$ Vertical bars represent standard errors of the means. Number of preparations used. cerebral 23; coronary 26; mesenteric arteries 27.

although the inhibition of the former contraction by $Cd^{2+}$ 5 μM was insignificant. After the $Ca^{2+}$-induced sustained contraction levelled off, cysteine (1 mM) or glutathione (1 mM) produced a relaxation in control preparations but a contraction in $Cd^{2+}$-treated arteries. In 8 out of 17 preparations treated with 20 μM $Cd^{2+}$, cysteine caused a biphasic pattern of contractions. Calcium ions (4.4 mM) failed to reverse the $Cd^{2+}$-induced inhibition. Typical recordings for the inhibitory effect of $Cd^{2+}$ and the reversal by cysteine are demonstrated in Figure 3. Prior treatment with cysteine (1 mM) completely prevented the inhibitory effect of 20 μM $Cd^{2+}$.

Treatment with $Cd^{2+}$ in concentrations higher than 20 μM caused a significant inhibition in the contractile response to $Ca^{2+}$ of coronary and mesenteric arteries (Table 1). Inhibition by 5 and 20 μM $Cd^{2+}$ was considerably less in coronary and mesenteric arteries than in cerebral arteries (Figure 4, left). Mean $ID_{50}$s in cerebral, coronary and mesenteric arteries were 6.2, 13.8 and 23.6 μM, respectively. The inhibitory effect of $Cd^{2+}$ was partially reversed by 1 mM cysteine.

### *Inhibition by verapamil of the $Ca^{2+}$-induced contraction*

Treatment for 20 min with verapamil in concentrations ranging from 50 nM to 1 μM caused a dose-related inhibition in both the phasic and sustained contractions induced by $Ca^{2+}$ in cerebral arterial strips exposed to $Ca^{2+}$-free media (Table 2). After $Ca^{2+}$-induced contractions levelled off, the addition of a further 4.4 mM $Ca^{2+}$ elicited a relaxation in control arteries but a contraction in verapamil-treated strips. Cysteine was ineffective in reversing the inhibitory effect of verapamil.

Verapamil in a concentration of 50 nM attenuated the contractile response of coronary arteries to $Ca^{2+}$ to an appreciably lesser extent than that of cerebral arteries, and this concentration of verapamil failed to attenuate significantly the response in mesenteric arteries (Table 2). Comparisons of the inhibitory effect of verapamil in these arteries is shown in Figure 4. Average $ID_{50}$s in coronary and mesenteric arteries were 0.1 μM and 0.14 μM, while the value in cerebral arteries was less than 50 nM.

### *Inhibition by papaverine of the* $Ca^{2+}$-induced contraction

Papaverine in concentrations of 1 to 20 μM caused a dose-dependent inhibition in the contractile response of cerebral and coronary arteries to 2.2 mM $Ca^{2+}$, while in mesenteric arteries, papaverine at 1 μM was ineffective and in concentrations higher than 5 μM significantly attenuated the $Ca^{2+}$-induced contraction (Table 3). Different susceptibility of the three arteries to papaverine is illustrated in Figure 4. $ID_{50}$s of cerebral, coronary and mesenteric arteries averaged 3.5, 4.9 and 12 μM, respectively.

## Discussion

The addition of $Ca^{2+}$ to isolated cerebral arteries of the dog exposed to $Ca^{2+}$-free media caused biphasic contractions. The transient contraction (independent of K⁺-induced depolarization) is possibly induced by influxes of $Ca^{2+}$ across cell membranes, in which the ion permeability increases following long exposure to $Ca^{2+}$-free media (Somlyo & Somlyo, 1968), while the sustained contraction may derive from slowly developing increase in $Ca^{2+}$ influxes in association with the membrane depolarization induced by elevated $[K^+]_o$. In the present study, the initial phasic contraction induced by $Ca^{2+}$ was markedly less and the contraction induced by K⁺ in $Ca^{2+}$-free media was greater in coronary and mesenteric arteries than in cerebral arteries. Membrane $Ca^{2+}$ plays a role in

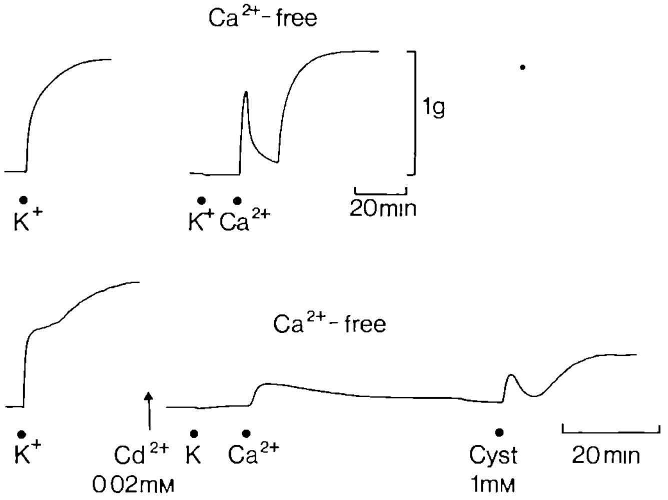

**Figure 3** Inhibitory effect of $Cd^{2+}$ on the $Ca^{2+}$-induced contraction in a basilar artery Two basilar arterial strips were obtained from the same dog, one for control series of experiments (upper tracings) and the other for experiments with 20 μM $Cd^{2+}$ (lower tracings). In these two strips, contractile responses to 25 mM $K^+$ were first obtained Preparations were then exposed for 60 min to $Ca^{2+}$-free media before the addition of $K^+$, in the lower tracings, $Cd^{2+}$ was added to the preparation after exposure for 40 min to $Ca^{2+}$-free media Cysteine (Cyst) produced a biphasic contraction after $Cd^{2+}$ treatment.

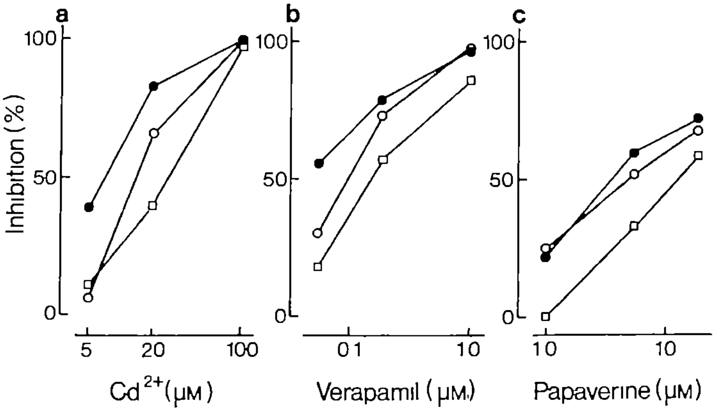

**Figure 4** Inhibition by (a) $Cd^{2+}$, (b) verapamil and (c) papaverine of the $Ca^{2+}$-induced contraction in cerebral (●), coronary (○) and mesenteric (□) arteries exposed to $Ca^{2+}$-free media. Each value was obtained from Tables 1, 2 and 3 by comparison of maximum contractions induced by $Ca^{2+}$ relative to those induced by $K^+$ in preparations exposed to control and experimental media (for instance, in cerebral arteries at 5 μM $Cd^{2+}$, 67% was expressed as a percentage of 110% and then subtracted from 100%).

stabilizing the membrane (Shanes, 1958) and is also released to cause a vascular contraction by the addition of $K^+$ (Somlyo & Somlyo, 1968). It appears that membrane $Ca^{2+}$ in cerebral arterial smooth muscle cells is easily depleted by 60 min exposure to $Ca^{2+}$-free media, as compared with that in coronary and mesenteric arteries, because the response to $K^+$ was greater in the latter.

Treatment with $Cd^{2+}$, like verapamil, a known $Ca^{2+}$ antagonist (Fleckenstein *et al.*, 1969) or papaverine, suppressed both the phasic and sustained contractions induced by $Ca^{2+}$ in cerebral arteries, indicating that the $Cd^{2+}$-induced inhibition does not derive from the antagonism to $K^+$-induced depolarization. Such non-selective inhibition by $Cd^{2+}$ of the contraction mediated via increased transmembrane influxes of

$Ca^{2+}$ is probably not due to interference with functioning of contractile proteins, since the contractile response of rabbit aortae to noradrenaline, histamine and angiotensin II is inhibited only slightly by $Cd^{2+}$ in concentrations sufficient to cause a marked attenuation of contractions induced by $K^+$ and $Ba^{2+}$ (Toda, 1973a). Further, contractility of the glycerinated aorta is unaffected by 100 µM $Cd^{2+}$ (unpublished data). Cysteine partially reversed and completely prevented the $Cd^{2+}$-induced inhibition seen in isolated aortae, atria and sinoatrial nodes of the rabbit (Toda, 1973a,b,c). It may therefore be concluded that $Cd^{2+}$ interferes with transmembrane influxes of $Ca^{2+}$ by a mechanism related to membrane SH groups. In fact, influxes of $^{45}Ca^{2+}$ measured by a lanthanum method (van Breemen, Farinas, Gerba & McNaughton, 1972) are significantly reduced by $Cd^{2+}$ (Toda, 1976a).

In canine isolated mesenteric arteries, $ID_{50}$s of verapamil and papaverine against $Ca^{2+}$ contractions were 0.14 and 12 µM respectively, and such data are consistent with the results obtained with rat isolated aortic ring preparations (Massingham, 1973). Contractile responses to $Ca^{2+}$ of cerebral and peripheral arteries of approximately the same size were suppressed in a different manner by vasodilator agents: susceptibility to $Cd^{2+}$ and verapamil was, cerebral > coronary > mesenteric, while that to papaverine was, cerebral = coronary > mesenteric. In contrast to the fact that isoproterenol, acetylcholine (Toda, 1974a) and dopamine (Toda, 1976b) which selectively stimulate respective drug receptors, cause considerably less relaxation in cerebral arteries than in mesenteric and coronary arteries, vasodilator agents acting directly on vascular smooth muscles appear to cause a greater relaxation in cerebral arteries.

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# THE GENERATION OF NERVE AND MUSCLE REPETITIVE ACTIVITY IN THE RAT PHRENIC NERVE-DIAPHRAGM PREPARATION FOLLOWING INHIBITION OF CHOLINESTERASE BY ECOTHIOPATE

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1 Simultaneous extracellular recordings were made from two end-plate zones of the isolated diaphragm and from the phrenic nerve of the rat in response to stimulation of the nerve. The contractions of the diaphragm were also recorded.

2 In the curarized diaphragm, the introduction of ecothiopate, a non-competitive inhibitor of cholinesterase, caused a threefold increase in the amplitude of the end-plate current and an eightfold increase in the duration at half the peak amplitude.

3 In the non-curarized diaphragm, the introduction of ecothiopate caused the generation of repetitive activity (RA) in first the phrenic nerve: this was then followed by RA in the diaphragm. At that stage, nerve RA possessed a shorter latency than muscle RA. The generation time for nerve RA was 1.6 ms and for mRA, it was 2.7 milliseconds.

4 Nerve RA was more labile than muscle RA; it was readily abolished by increasing the frequency of stimulation, by magnesium, by tubocurarine or by high concentrations of ecothiopate, whereas muscle RA was still generated. Steady exposure to acetylcholine abolished both forms of RA.

5 Two competitive inhibitors of cholinesterase, neostigmine and ambenonium, were also shown to evoke RA in nerve and muscle. The generation times for nerve RA and muscle RA were similar to those following ecothiopate.

6 It was concluded that nerve RA and muscle RA were generated after the inhibition of cholinesterase by ecothiopate as a result of the prolonged action of acetylcholine upon cholinoceptive sites on the nerve terminal and motor endplate respectively. A direct excitatory action of ecothiopate upon the phrenic nerve terminals was excluded.

## Introduction

After physostigmine, stimulation of the motor nerve evokes repetitive activity in the skeletal muscle of cat (Brown, Dale & Feldberg, 1936). Masland & Wigton (1940) showed that, after neostigmine, antidromic activity also occurs in the ventral roots of the spinal cord. They concluded that, after inhibition of cholinesterase (ChE), the prolonged action of acetylcholine (ACh) upon cholinoceptive sites on the motor nerve terminal and motor endplate generated nerve repetitive activity (nRA) and muscle repetitive activity (mRA), respectively. In contrast, Eccles, Katz & Kuffler (1942) and Lloyd (1942) proposed that mRA was generated by the prolonged action of ACh at the motor endplate and this activity was conducted retrogradely to evoke nRA in the nerve terminal. A third proposal is that nRA is generated as a result of the direct action of the ChE inhibitor upon the nerve terminal. The generation of nRA, in turn leads to further ACh release from the nerve terminal and so gives rise to mRA (Riker & Okamoto, 1969; Riker, 1975). Inhibition of ChE was not considered to be an essential step in the generation of RA (Blaber, 1972).

The intention of the present study was to determine which of these proposals is correct. A preliminary account of this work has been presented (Morrison, 1976).

## Methods

The methods used in this study are described more fully by Ferry & Marshall (1973). Hemi-diaphragms from albino rats, 150–250 g in weight, were superfused at 3 ml/min *in vitro* with physiological saline (composition (mM): NaCl 137, KCl 5, $NaHCO_3$ 12, $NaH_2PO_4$ 1.0, $MgCl_2$ 0.1–1.0, $CaCl_2$ 2.0 and glucose 25) maintained at 37°C and gassed with 5% $CO_2$ in $O_2$. To facilitate a rapid change, the dead space in the apparatus was flushed through with fresh solution: then rapid flow over the preparation was allowed for a short period. Elapsed time was measured from the introduction of the new solution.

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Simultaneous extracellular recordings were made in response to supramaximal stimulation of the phrenic nerve by 2 V pulses, 50 µs duration and frequency 0.1 hertz. Focal extracellular recordings were made from two end-plate zones (M1 and M2). The electrodes consisted of insulated silver wire, 125 µm in diameter, cut cleanly at right angles with a razor blade and supported in glass micropipettes. The recording system had a bandwidth of 10 kHz–0.8 Hz (–3 db). To locate an end-plate zone, the electrode was placed over a nerve terminal (but not in contact with the diaphragm) so that the negative spike with fastest rise time was recorded. At high gain, the positive-negative pre-synaptic spike was visible. The negative-positive muscle spike was superimposed upon the end-plate current (e.p.c.) and their summation frequently resulted in a complex waveform (see later). Small changes in the electrode's position with respect to the tissue occurred during the experiments so adjustments were made regularly to keep the recorded waveform constant. The prolongation of the e.p.c. after ecothiopate confirmed that the end-plate zone had been located. The 'matched-pair' preparation in which recordings were taken from a single nerve fibre and from the muscle fibre which it innervated (Standaert, 1963; Riker, 1966) was not employed but precautions were taken to validate comparisons between nerve and muscle recordings. The two end-plate recordings M1 and M2 were always very similar, despite having been made on opposite sides of the preparation, and were taken to be representative of activity throughout the diaphragm. In some experiments, branches of phrenic nerve, innervating parts of the diaphragm not recorded from, were sectioned. The contraction was recorded isometrically and was displayed upon a Devices M2 recorder or oscilloscope. The activity of the phrenic nerve was recorded in air with silver wire electrodes with bandwidth 40 kHz–40 Hz (–3 db). All responses were displayed upon a Tektronix 564B storage oscilloscope and photographed.

Drugs used were acetylcholine chloride (BDH); ecothiopate iodide (Ayerst Laboratories); tubocurarine chloride, neostigmine methyl sulphate (Koch-Light Laboratories); and ambenonium chloride (Sterling-Winthrop).

## Results

Experiments were performed upon 142 hemidiaphragms (both left and right). The main ChE inhibitor used in this study was ecothiopate, an agent used in eye drops for the relief of glaucoma. It is safe to use and is easily obtainable. Ecothiopate inhibits ChE irreversibly: the reaction follows pseudo first order kinetics (Ferry & Marshall, 1971; Ferry & Marshall, unpublished observations). Two competitive inhibitors of ChE, neostigmine and ambenonium, were also tested.

### *Effect of ecothiopate upon the curarized preparation*

In the preliminary experiments the diaphragm was exposed to tubocurarine 3.0 µM in saline for one hour to give a stable block of contraction. The e.p.c. was recorded focally at high gain in response to stimulation of the phrenic nerve. In the presence of ecothiopate 500 nM, the amplitude of the e.p.c. was increased 1.2–5.1 (mean 2.5) times and the duration at half amplitude was increased 5.6–14.2 (mean 8.1) times: from 0.66 ± 0.08 ms (mean ± s.e. mean) to 5.42 ± 0.47 milliseconds. The changes were well developed after 10 min and reached a maximum after 30 min: they were not affected by removal of ecothiopate.

By the use of photographic enlargements, the transmission time across the neuromuscular junction was measured from the negative peak of the presynaptic spike to the start of the rise of the e.p.c., in five preparations. Transmission time was 0.15 ± 0.008 ms (mean ± s.e. mean). This agrees well with the value of 0.22 ms given by Hubbard & Schmidt (1963). Within the limitations of the measurements, there appeared to be no difference in the transmission time before and after the addition of ecothiopate.

### *Effect of ecothiopate upon the non-curarized preparation*

After preliminary experiments upon the non-curarized diaphragm using normal physiological saline solution (in which only low levels of nRA were generated), the $Mg^{2+}$ concentration of the saline was reduced from 1.0 to 0.1 mM in order to facilitate the generation of nRA and mRA (see later). Firstly, mRA is described. Control records from M1 and M2, are shown in Figure 1A. In these traces, the stimulus occurred at the start of the sweep while in other records (e.g. Figure 3) the stimulus artefact was present. The negative muscle spike (a) generated within the end-plate zone was followed by a positive phase (the presynaptic spike is not visible in records at low gain). The muscle spike had such a fast rise time that it was incompletely stored in many traces. After the introduction of 50 nM ecothiopate, progressive prolongation of the e.p.c. (c) occurred in both M1 and M2 (Figure 1B and C). After 17 min, mRA (m) was present, always superimposed upon the prolonged e.p.c. in M1 and M2 (Figure 1D). After longer

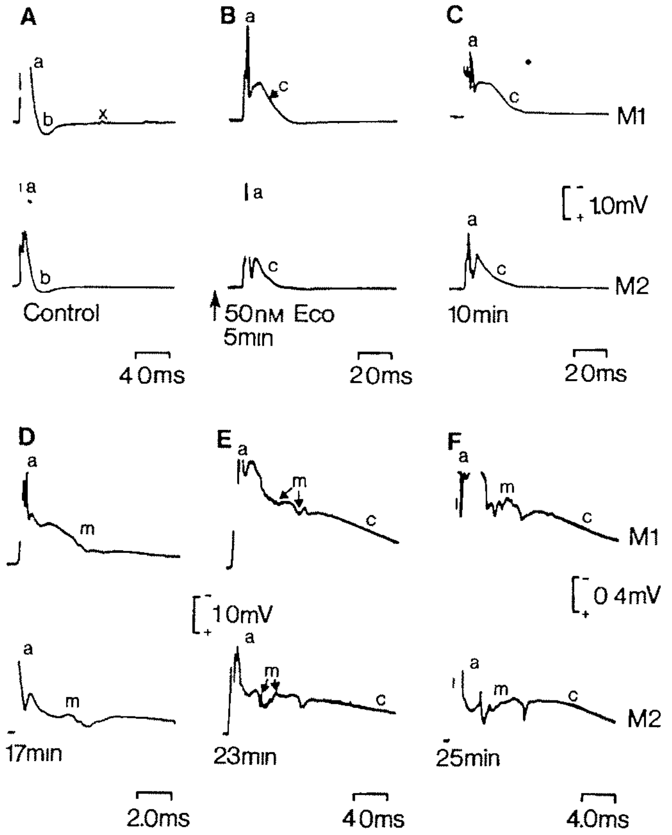

**Figure 1** Simultaneous focal extracellular recordings from two end-plate zones M1 (upper trace) and M2 (lower trace). The muscle spikes were incompletely stored due to their fast rise time. (A) Control records showing the negative spike (a) followed by the positive undershoot (b) Blips on the baseline of M1 (x) were peculiar to that sweep (B) After 5 min in 50 nM ecothiopate (Eco). Slight prolongation of the e.p.c (c). (C) After 10 min, further prolongation of the e.p c. (D) After 17 min, first appearance of muscle repetitive activity mRA (m). (E) After 23 min marked increases in mRA and in duration of the e.p.c. (F) After 25 min, mRA now quite pronounced. Note how M1 and M2 develop in parallel. Amplification was increased in (E) and (F)

exposures to ecothiopate, mRA increased in magnitude (Figure 1E and F). The possibility of movement artefacts in the traces was excluded when the electrode was placed near the lateral edge of the diaphragm where movement but not contraction occurred (movement was due to contraction of the main central part of the diaphragm): no movement artefacts were recorded.

The changes in the records at M1 and M2 developed in parallel (Figure 1). Thus M1 and M2 were taken to represent activity throughout the diaphragm and were compared with the records of activity in the phrenic nerve even though this may not have been generated at the two actual end-plate zones which were monitored.

Ecothiopate also increased the amplitude and duration of the contraction of the diaphragm. Furthermore the diaphragm relaxed very slowly after contraction had occurred. Figure 2 illustrates the effects of 500 nM ecothiopate: similar results were observed with 50 nM ecothiopate, except that the effects took longer to develop. After 9 min, the amplitude of the contraction had increased (but later began to decrease) (Figure 2B) and there was a persistent prolongation of relaxation (Figure 2C). Brown *et al.* (1936), using physostigmine, had noted the increased twitch duration but not the prolonged relaxation.

### *Simultaneous recordings from the diaphragm and phrenic nerve*

Recordings were made simultaneously from M1, M2 and N in 0.1 mM $Mg^{2+}$ saline. After the introduction

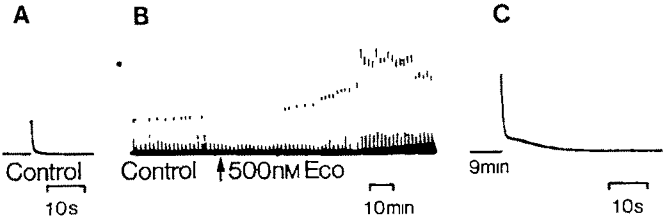

Figure 2 Effects of 500 nM ecothiopate upon isometric recordings of the contraction of the diaphragm. (A) Control contraction (B) Introduction of 500 nM ecothiopate (Eco) A marked increase in amplitude had occurred. (C) After 9 min in ecothiopate Prolongation of relaxation time had occurred The amplitude of the contractions was uncalibrated.

of ecothiopate, nRA appeared within 12–20 min at 50 nM, 8–10 min at 100 nM and 4–6 min at 500 nM ecothiopate. Muscle RA started several minutes later in each case. With 5 nM ecothiopate no perceptible changes occurred even after an hour. After ecothiopate, spontaneous activity in the phrenic nerve was never observed. The antidromic discharges in response to stimulation consisted of 2–20 spikes in a train of duration less than 10 ms and with a very high frequency. The stages in the generation of RA were examined when exposures to 100 nM ecothiopate lasting 2–3 min were alternated with irrigations with saline lasting 10–15 minutes. Figure 3 illustrates part of a typical experiment. The nerve recordings may be divided into several parts. The first is the stimulus artefact (d) which was incompletely stored: it also obscured the orthodromic action potential in most records. The peak referred to as the 'electrotonic potential' (e) reflected the amplitude of the muscle activity and was thought to be the latter conducted over the surface of the phrenic nerve. However it is not a reliable indication of diaphragm activity since the conducting properties of the nerve's surface declined as the nerve progressively dried out during the experiment. For this reason, the peak e was much smaller in Figure 3C and D. Nerve RA is labelled n. The possibility of movement artefacts affecting the nerve recordings may be discounted since the contraction did not occur until after the start of nRA.

The control records in Figure 3A reveal no sign of RA in either nerve or muscle. After 3 exposures to ecothiopate which gave a total exposure time of 7 min, nRA was recorded but mRA was not (Figure 3B). After further exposure to ecothiopate for 4.5 min, mRA (m) appeared. By this time, there was marked prolongation of the e.p.c. and the nRA was well developed (Figure 3C). After further exposure to ecothiopate, nRA, mRA and the e.p.c. increased (Figure 3D). The latencies for the generation of nRA and mRA were measured from the stimulus artefact when nRA and mRA had just become well developed (at a slightly later stage than shown in Figure 3C), and

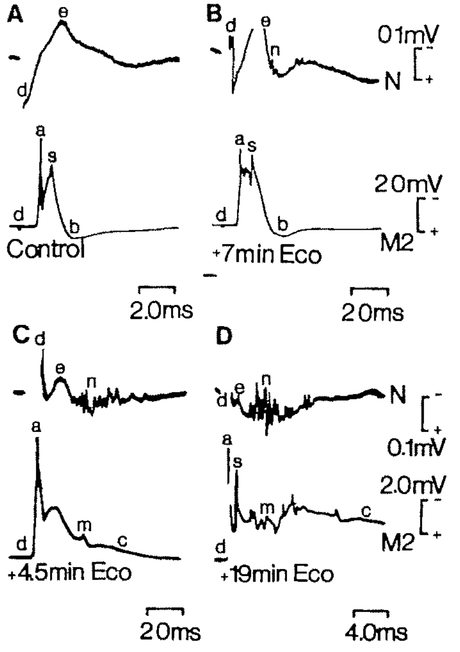

Figure 3 Simultaneous recordings from N (upper trace) and M2 (lower trace) after short exposures to 100 nM ecothiopate (Eco) followed by periods of irrigation with saline. (A) Control records: M2 shows the negative spike (a) and a smaller secondary spike (s), perhaps arising from the asynchronous discharge of muscle fibres, followed by a positive undershoot (b) N shows the incompletely stored stimulus artefact (d) followed by the electrotonic potential (e). (B) After 7 min in ecothiopate: appearance of nerve repetitive activity, nRA (n) but muscle repetitive activity (mRA) not present. (C) After a further 4.5 min in ecothiopate: prolongation of the e.p.c (c) and appearance of mRA (m); nRA appreciably increased. (D) After a further 19 min in ecothiopate: both nRA and mRA were well developed and the e.p c. was very prolonged In (D) trace speed was halved.

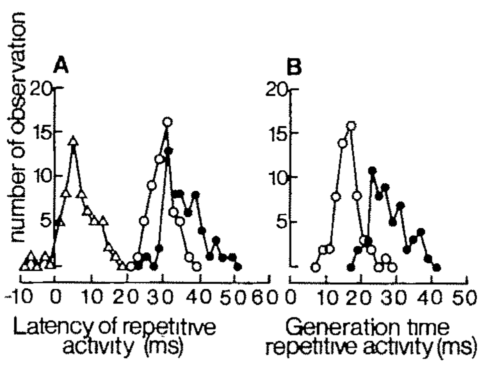

**Figure 4** (A) Latencies for nerve repetitive activity (nRA) and muscle repetitive activity (mRA), after the introduction of 100 nM ecothiopate: (○) nRA, (●) mRA; (△) differences between mRA and nRA (for positive intervals nRA preceded mRA). (B) Generation times. (○) nRA, (●) mRA. Further explanation is given in the text.

are presented in Figure 4A: mean values were 2.94 ± 0.05 ms for nRA and 3.56 ± 0.08 ms for mRA. Nerve RA preceded mRA in all but 2 out of 57 diaphragms: the mean interval was 0.69 ± 0.06 milliseconds. These values did not contain a correction for conduction in the phrenic nerve (but see later). For longer exposures to ecothiopate, the nRA latency increased progressively to 6–8 ms while the latency for mRA remained unchanged. With 500 nM ecothiopate, the nRA latency was also 6–8 ms which exceeded the mRA latency of 3–4 milliseconds.

The generation time for nRA was the interval between the invasion of the nerve terminal by the orthodromic impulse and the generation of the first antidromic impulse. The generation time for mRA was the interval between the stimulus-evoked muscle spike and the foot of the first repetitive spike. These two values were calculated for 56 preparations. The generation time for nRA was calculated from the latency for nRA, as follows. The interval between the stimulus and the muscle spike comprised the conduction time in the phrenic nerve and the transmission time across the neuromuscular junction (0.15 milliseconds). So the nerve conduction time was the muscle spike latency less 0.15 milliseconds. Then, twice the conduction time (for conduction down the nerve and the return) was subtracted from the nRA latency to yield the generation time. (These calculations did not take into account the fact that conduction from the nerve terminal was to the recording electrode and not to the stimulating electrode nor that recent activity in the nerve may have reduced the antidromic conduction time, see Waxman & Swadlow, 1976; however, both corrections were very small.) The distribution of generation times for nRA is presented in Figure 4B. The mean value was 1.58 ± 0.05 ms which exceeds the value of 1.0 ms obtained by Werner (1961) in the cat, but agreed well with the value of 1.5 ms determined for the absolute refractory period of phrenic nerve terminals (Hubbard & Schmidt, 1963).

The generation time for mRA was calculated by the subtraction of the muscle spike latency from the mRA latency and the distribution is presented in Figure 4B: the mean value was 2.74 ± 0.08 ms which was appreciably longer than the value for nRA. This value exceeds the absolutely refractory period of mammalian striated muscle, 2 ms (Bowman, Rand & West, 1969). However, since diaphragm muscle may differ from other skeletal muscle in its refractory period, a comparison based upon an absolute refractory period of 2 ms is of limited value. It is of interest that, as the experiment progressed, the generation time for nRA progressively increased whilst for mRA it remained the same. This is relevant to the next section which describes the inhibitory effect which high concentrations of ecothiopate exerted upon nRA.

### *Effects of high concentrations of ecothiopate*

After 500 nM ecothiopate nRA and mRA appeared within 4–8 min, the nRA latency gradually increased and the magnitude of the nRA gradually declined until it eventually disappeared 15–20 min after the introduction of ecothiopate. This occurred in 16 out of 18 experiments. Then, when the diaphragm was superfused with saline, nRA returned within 5–10 minutes. Muscle RA remained unaffected. A preparation treated with 50–100 nM ecothiopate developed nRA and mRA within 10–20 minutes. If the ecothiopate was washed out, the RA remained fairly stable, declining only slowly. When 500 nM ecothiopate was then added to such a preparation, there was suppression of nRA but no effect on mRA (9 out of 11 experiments). So it is suggested that ecothiopate in concentrations of 500 nM exerts a reversible inhibition of the appearance of nRA.

### *Effects of frequency of stimulation upon repetitive activity*

The abolition of nRA by increasing the frequency of stimulation was a convenient means of checking that the recordings were not due to fluctuations in the baseline. When 'stable' RA had appeared (after 50 or 100 nM ecothiopate which was applied and then washed out) nRA was depressed when the stimulation frequency was increased to 1.0 Hz and abolished at 2.0 Hz or, in exceptional circumstances, 3.3 hertz. Under these conditions, mRA was only reduced; but was abolished at 5–10 hertz. The presence of 500 nM ecothiopate greatly increased the susceptibility of nRA

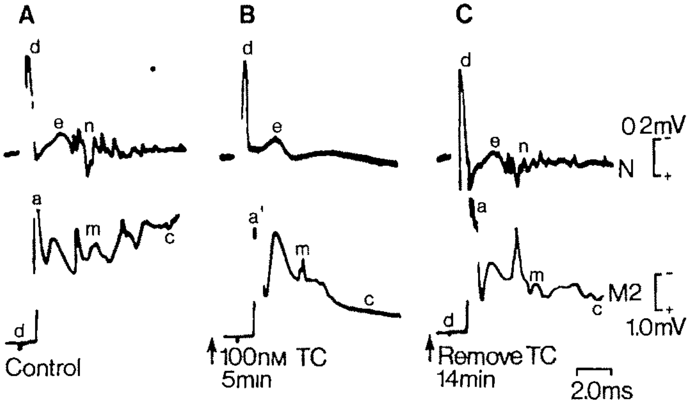

**Figure 5** Effects of tubocurarine (TC) upon N (upper trace) and M2 (lower trace). Ecothiopate was removed after repetitive activity (RA) had developed. (A) Control records N consists of stimulus artefact (d), electrotonic potential (e) and well developed nRA (n), while in M2 the e.p.c. (c) was very prolonged and mRA (m) was present. (B) After 5 min in 100 nM tubocurarine: nRA was abolished, mRA was depressed and the e.p.c. was reduced. (C) Return of nRA and mRA, 14 min after irrigation with saline. Note that the e.p.c. did not return to its control level

to depression when 0.3 Hz or 0.5 Hz were quite sufficient to abolish nRA whilst mRA was undiminished.

*Effects of magnesium upon repetitive activity*

When the $Mg^{2+}$ concentration was increased from 0.1 to 1.0 or 2.0 mM, nRA was abolished while mRA was only reduced and the duration of the e.p.c. was shortened. The effects of the $Mg^{2+}$ were fully developed after about 10 min and were reversed over a similar period after removal of $Mg^{2+}$. These experiments demonstrated that mRA was recorded in the absence of nRA. The effects of the increased $Mg^{2+}$ might be due to reduced ACh release (Liley, 1956). However, increased $Mg^{2+}$ also stabilizes the neurolemma (Frankenhaeuser & Hodgkin, 1957); but this was unlikely to be important since reduced $Ca^{2+}$ (0.2 mM) saline, which would labilize the membrane, also depressed both nRA and mRA.

*Effects of tubocurarine upon repetitive activity*

In concentrations much lower than those required to abolish the contraction of the diaphragm, tubocurarine depressed RA (Figure 5). Within 5–10 min of its introduction at 100 nM, mRA and the e.p.c. were both depressed while nRA was abolished (Figure 5B). Irrigation with saline for 5–10 min restored the nRA and mRA, although the e.p.c. had not returned to its control duration (Figure 5C). These results showed that, once again, it was possible to record mRA in the absence of nRA. (The failure of the e.p.c. to return to its control duration suggested that tubocurarine was still bound to cholinoceptive sites on the motor endplate despite prolonged irrigation with saline.)

*Effects of acetylcholine upon repetitive activity*

Introduction of 10 μM ACh had little effect upon the nRA and mRA evoked by stimulation of the phrenic nerve, whilst 55, 100 or 110 μM ACh abolished both nRA and mRA within 5–10 minutes. It was not possible to discern if one type of RA was more susceptible than the other. Both nRA and mRA returned after irrigation with saline for 8–10 minutes.

*Effects of neostigmine and ambenonium*

In order to see whether the results obtained with ecothiopate applied to other ChE inhibitors, two competitive inhibitors of ChE, neostigmine and ambenonium, were tested. On a molar basis both agents were more effective in the generation of RA than ecothiopate. The onset of nRA and mRA and prolongation of the e.p.c. occurred within 4 min of the introduction of 100 nM neostigmine or 100 nM ambenonium. These effects were well sustained for that concentration, as illustrated by Figure 6 for neostigmine. Irrigation with saline removed nRA and mRA. The e.p.c. was reduced in duration but was still very prolonged compared with the duration before neostigmine.

After the introduction of neostigmine or ambenonium, when nRA and mRA were well developed, the latency of nRA was less than the latency of mRA, and the generation times for nRA

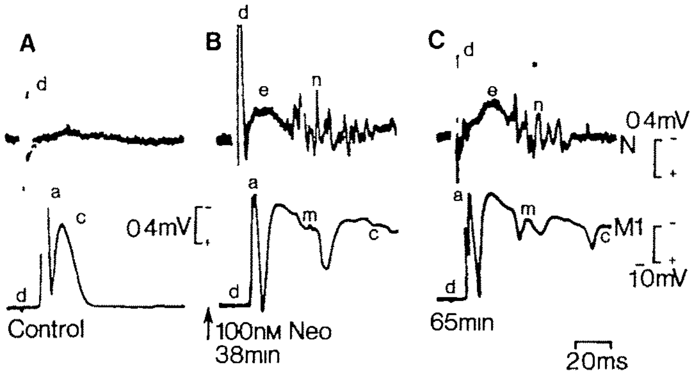

**Figure 6** Effects of neostigmine upon N (upper trace) and M1 (lower trace). (A) Control records. N shows stimulus artefact (d) but electrotonic potential (e) was not visible M1 shows muscle spike superimposed upon the e.p.c. (c). (B) After 38 min in 100 nM neostigmine (Neo): nerve repetitive activity, nRA (n) and muscle repetitive activity, mRA (m) were well developed and the e.p.c. was markedly prolonged. (C) After 65 min in neostigmine. Both nRA and mRA and prolongation of the e p c were sustained Amplification of M1 was reduced in (B) and (C).

and mRA were 1.3 ms and 2.5 ms respectively with neostigmine and 1.8 ms and 2.4 ms respectively with ambenonium: these agree well with the results for ecothiopate. When 3.3 µM neostigmine was introduced nRA and mRA were generated within seconds but several minutes later, nRA had disappeared whilst mRA was undiminished. Similar results were obtained with 500 nM neostigmine. Removal of neostigmine brought some but not complete return of nRA. Ambenonium differed from neostigmine since, after the introduction of 1.0 µM ambenonium, nRA and mRA were sustained throughout the experiment.

It may be concluded that the actions of neostigmine and ambenonium in the generation of RA were similar to those of ecothiopate.

## Discussion

In the present study electrical activity at the end-plate zone has been related to antidromic activity in the phrenic nerve, after treatment of the diaphragm with ecothiopate. Some limitations of this work are as follows:

(1) Antidromic activity recorded in the phrenic nerve might not have arisen from the nerve terminals from which end-plate activity was recorded. However comparisons between the nerve and muscle recordings were in part justified by demonstrating that the two end-plate recordings M1 and M2 were similar. Hence they were presumed to be representative of end-plate activity throughout the diaphragm.

(2) Low $Mg^{2+}$ saline was used in the present study and so the results are not directly applicable to the intact animal. The effects of reducing the $Mg^{2+}$ may be to increase ACh release (Liley, 1956) and so the difference may be quantitative rather than qualitative. Nevertheless the results do give insight into the generation of nRA, which would not have been possible if normal $Mg^{2+}$ had been used. Barstad (1962), Randić & Straughan (1964) and Blaber (1972) also used low $Mg^{2+}$ solutions.

(3) The absolute sensitivities of the nerve and muscle recording electrodes are not known, so low levels of nRA and mRA may not have been detected. End-plate activity was also usually recorded at a lower gain to ensure that the recording was always focal.

(4) The electrodes M1 and M2 may have obstructed the diffusion of ACh from the end-plate zone and thus artificially prolonged the e.p.c. (Katz & Miledi, 1973). However the electrodes were placed above the nerve terminals and not actually in contact, so this effect is believed to be minimal.

(5) An *in vitro* preparation was employed. After several hours, deterioration of the preparation was readily observed by marked falls in the amplitudes of the end-plate recordings. For this reason experiments were performed usually within an hour of setting up the preparation. The nerve was stimulated only when recordings were taken.

Within these limitations, the present work has demonstrated the independence of nRA and mRA. Nerve RA had a shorter generation time than mRA and in the early stages of the action of ecothiopate low levels of nRA were recorded in the absence of mRA. This suggests that nRA was not generated by mRA. Since nRA was readily abolished whilst mRA was still recorded, nRA *per se* did not generate mRA. Prolongation of the e.p.c. accompanied nRA and mRA (cf. Blaber, 1972) but the e.p.c. *per se* was unlikely to have generated nRA (though it generated mRA) because this would require retrograde conduction across the neuromuscular junction. This is improbable since repetitive postsynaptic activity depressed pre-synaptic activity, perhaps by accumulation of extracellular $K^+$ (Weight & Erulkar, 1976). So the prolonged action of ACh is proposed to be the generator of both nRA and of the prolonged e.p.c. and associated mRA. Experiments which reduce the amount of ACh released from the nerve terminal (increasing $Mg^{2+}$ or frequency) or compete with the binding of ACh to cholinoceptive sites (tubocurarine) reduce nRA and mRA. Generally nRA was more labile than mRA. This may be attributable to a lower density of cholinoceptive sites on the nerve terminal than on the motor end-plate. This proposal would mean the probability of an ACh molecule binding with a receptor site was lower for the nerve terminal than motor end-plate. So the nerve terminal would be more sensitive to reduced ACh release. The proposal of Riker (1975) that the ChE inhibitor generates nRA directly, as opposed to indirectly via excess ACh, appears unlikely for several reasons.

(1) The present study showed that nRA was generated after ecothiopate had been washed out (although there is the possibility of ecothiopate binding to the nerve terminal).

(2) Muscle RA may be recorded in the absence of nRA. This has even been described by Riker and his associates in matched-pair experiments (Standaert & Adams, 1965; Riker, 1966). To account for this Riker & Okamoto (1969) proposed that antidromic nRA is more easily abolished than orthodromic nRA from the sites of initiation. While this proposal is difficult to refute, no evidence in support of it has been presented and so it appears reasonable to accept the results at face value, that in fact, no nRA was generated in the nerve terminal.

(3) If nRA arose as a result of the increased excitability of the nerve terminal due to the direct action of ecothiopate, nRA would be expected to be generated within the subnormal period which extended from 10 to 20 ms after orthodromic stimulation (Hubbard & Schmidt, 1963). However, the present results suggested that nRA was generated immediately after the absolute refractory period which was up to 1.5 ms after orthodromic stimulation (Hubbard & Schmidt, 1963).

In conclusion, if nRA and mRA both result from the prolonged action of ACh following ChE inhibition (as we suggest) it is no surprise that synchrony between nRA and mRA occurred in matched-pair experiments. However, nRA and mRA are nonetheless independent and thus one form of RA may be recorded in the absence of the other.

The provision of equipment by the Muscular Dystrophy Group of Great Britain and The Boots Company, and the loan of apparatus by the Department of Pharmacology, University of Newcastle upon Tyne is gratefully acknowledged. I also wish to thank Professors W.C. Bowman and O.F. Hutter for valuable discussion and, in particular, Professor C.B. Ferry for his interest in this work and for the provision of equipment and facilities.

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# POSSIBLE MECHANISM OF ADVERSE REACTION FOLLOWING LEVODOPA PLUS BENSERAZIDE TREATMENT

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1 Rats treated for seven days with seryl-trihydroxybenzylhydrazine (benserazide), an inhibitor of peripheral aromatic L-amino acid decarboxylase (500 mg/kg, daily, i.p.) alone or in combination with L-DOPA methylester (500 mg/kg, daily, i.p.) for seven days showed a moderate but significant decrease of liver aldehyde dehydrogenase (ALDH), without accompanying change in alcohol dehydrogenase (ADH) activity, compared with saline-treated controls.

2 Administration of L-DOPA methylester (500 mg/kg, daily, i.p.) alone for seven days had little effect on liver ADH or ALDH.

3 The combined treatment might be conducive to the *in vivo* formation of L-DOPA-derived tetrahydroisoquinoline derivatives which might be implicated in L-DOPA produced adverse effects.

## Introduction

The clinical efficacy of 3,4-dihydroxyphenylalanine (L-DOPA) in the treatment of Parkinson's disease is well established (Cotzias, Van Woert & Schiffer, 1967). However, L-DOPA produced dose-dependent side-effects often limit the desired therapeutic response. Co-administration of L-DOPA with an inhibitor of peripheral aromatic L-amino acid decarboxylase (ADC), e.g. benserazide (Birkmayer, 1969; Tissot, Gaillard, Guggisberg, Gautier & de Ajuriaguerra, 1969) or carbidopa (Cotzias, Papavasiliou & Gellene, 1969), reduced the therapeutic dose requirements for L-DOPA and decrease some of L-DOPA mediated adverse effects. The search for biochemical explanations for L-DOPA-induced side-effects resulted in the hypothesis (Sandler, 1973) that tetrahydroisoquinoline derivatives formed during L-DOPA therapy (Sandler, Carter, Hunter & Stern, 1973; Davis, Cashaw & McMurtrey, 1975), from the condensation of L-DOPA-derived monoamines with their respective oxidative deaminated metabolites (Holtz, Stock & Westermann, 1964; Collins, Cashaw & Davis, 1973; Turner, Baker, Algeri, Frigerio & Garattini, 1974), might be implicated in L-DOPA-induced adverse reactions. Furthermore, it is known that inhibition of aldehyde dehydrogenase (ALDH) by certain drugs augments the formation of these alkaloid-like compounds which may possess considerable pharmacological activity (Holtz *et al.*, 1964; Baird-Lambert & Cohen, 1975).

Benserazide possesses a terminal alcohol group which can be oxidized to a corresponding aldehyde derivative; subsequent cleavage of the benserazide molecule by hydrolysis of the seryl-hydrazine linkage (Bukard, Gey & Pletscher, 1964) may well produce alcohol and aldehyde intermediates capable of altering the activities of both alcohol dehydrogenase (ADH) and ALDH. To test this possibility, the specific activities of liver ADH and ALDH were determined after acute and short-term administration of benserazide and L-DOPA to rats.

## Methods

Adult male Sprague-Dawley rats, 70–85 day old, were maintained on Purina pellet food and water *ad libitum*. They were divided into eight groups, four groups each of the acute and semi-chronic experiments. Drugs were all administered intraperitoneally (i.p.) in 0.9% w/v NaCl solution (saline). In the acute experiments, one group was given benserazide ($N^1$-DL-seryl-$N^2$-(2,3,4 trihydroxybenzyl) hydrazine, Ro 4-4602), 500 mg/kg body weight; another received L-DOPA methylester 500 mg/kg; the remaining two groups were injected with a combination of benserazide and either L-DOPA methylester (500 mg/kg each) or saline. In the semi-chronic experiments, the same drugs in identical dosages were administered once daily for seven consecutive days. All animals were killed by decapitation 16 h after drug administration; their livers were removed, rinsed with 0.1 M phosphate buffer, pH 7.0, weighed individually; homogenized in sufficient ice-cold 0.1 M KCl solution to make a 15% (w/v) homogenate and centrifuged for 90 min at 22,000 *g*. The resulting supernatants were assayed for cytoplasmic ADH (EC, NAD, 1.1.1.1) and ALDH (EC, NAD, 1.2.1.3.) by the methods of Blair & Vallee (1966) and Blair & Bodley (1969), respectively. A portion of each supernatant fluid was utilized for protein determination by the biuret procedure. Findings are expressed in terms of specific activity (nmol $min^{-1}$ $mg^{-1}$ protein) measured at 25°C. The data were evaluated by Student's *t*-test.

## Results and Discussion

Figure 1 shows the effects of acute (upper panel) and semi-chronic administration (lower panel) of benserazide and L-DOPA, separately and in combination on specific activities of liver ADH and ALDH, expressed as mean ± s.d. Acute administration of the drugs produced little change in specific activity of either enzyme compared with saline-treated controls. However, benserazide, 500 mg/kg daily for seven consecutive days, brought about a moderate (19.6%) decrease of liver ADH compared with control value although this did not reach significance ($P < 0.1$). At the same time, there was a significant (25.5%) inhibition of liver-ALDH activity compared with saline-treated rats ($P < 0.01$). Semi-chronic administration of L-DOPA alone did not alter liver ADH and liver-ALDH activities. Co-administration of benserazide with L-DOPA for seven days significantly decreased ALDH activity to a mean of 4.13 ± 0.51 nmol $min^{-1}$ $mg^{-1}$ protein compared with 6.04 ± 0.38 nmol $min^{-1}$ $mg^{-1}$ protein for saline controls ($P < 0.05$).

The present results show that semi-chronic administration of benserazide, alone or in combination with L-DOPA, results in some inhibition of liver ADH and significant reduction in liver-ALDH activities. It should be noted that intermediate aldehydes derived from the oxidative deamination of monoamines are reduced to their corresponding alcohol metabolites by the action of ADH or oxidized to acid derivatives by ALDH. These pathways represent the major routes of metabolism for aldehyde intermediates of catecholamines in both extracerebral tissues and brain (Taylor & Laverty, 1969) respectively. It is thus conceivable that inhibition of liver ALDH in a manner noted here by an inhibitor of peripheral ADC accompanying L-DOPA therapy might give rise to condensation products composed of DOPA-derived aldehydes and their amine precursors. These compounds may act as false transmitters (Cohen, 1973) and in this way contribute to the appearance of L-DOPA-induced adverse reactions.

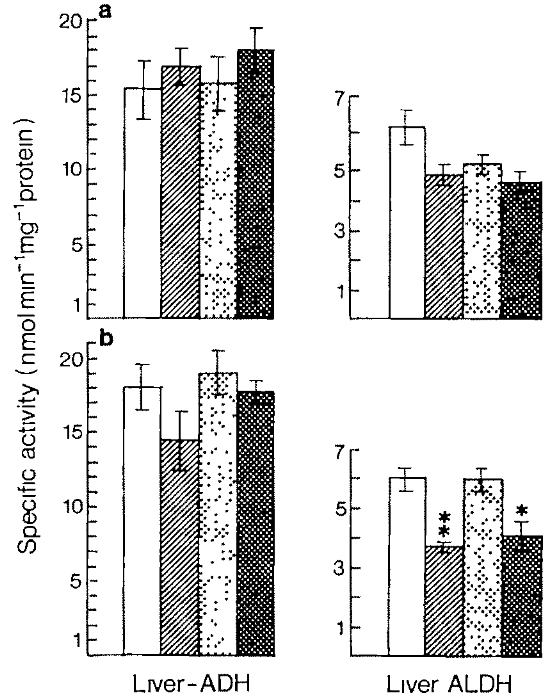

**Figure 1** Effect of (a) acute and (b) semi-chronic administration of L-DOPA methylester (500 mg/kg, i.p.), benserazide, an inhibitor of aromatic L-amino acid decarboxylase (ADCI), (500 mg/kg, i.p.) separately and together, on the specific activities (nmol $min^{-1}$ $mg^{-1}$ protein) of rat liver alcohol dehydrogenase (ADH) and liver aldehyde dehydrogenase (ALDH). Each value represents the mean derived from eight rats in each treatment group. Vertical lines show s.d. Open columns = saline-treated controls; hatched columns = ADCI-treated; strippled columns = L-DOPA-treated; cross-hatched columns = ADCI plus L-DOPA-treated. *$P < 0.05$; **$P < 0.01$.

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# DEVELOPMENT OF NICOTINIC RESPONSES IN THE RAT ADRENAL MEDULLA AND LONG-TERM EFFECTS OF NEONATAL NICOTINE ADMINISTRATION

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1 The development of nicotinic responses in the rat adrenal medulla was examined at various ages from 1 to 50 days of age by testing the ability of nicotine (10 mg/kg, s.c.) to deplete catecholamines and induce tyrosine hydroxylase.

2 Catecholamines were depleted 25% 3 h after injection of nicotine at all ages tested, but the degree of tyrosine hydroxylase induction 24 h after nicotine increased with age.

3 These data indicate that functional nicotinic receptors are present in the neonatal adrenal medulla before the development of functional splanchnic innervation, but that the development of the ability to induce tyrosine hydroxylase is not coupled directly to the development of secretory mechanisms.

4 The long-term effects of a single dose of nicotine (10 mg/kg, s.c.) administered to one day old rats were also examined.

5 After the short-term catecholamine depletion caused by nicotine, there were persistent elevations of catecholamines and tyrosine hydroxylase until 23 days of age; however, dopamine $\beta$-hydroxylase remained elevated into young adulthood.

6 These data indicate that neonatal nicotine administration can produce long-term changes in adrenal catecholamine biosynthetic enzymes.

## Introduction

Nicotine stimulates the adrenal medulla by at least three distinct mechanisms: direct stimulation of nicotinic receptors in the chromaffin cells (Schneider, 1969), stimulation *via* sympathoadrenal reflexes (Patrick & Kirshner, 1971a), and, to a lesser degree, hormonal stimulation from the adrenal cortex (Rubin & Warner, 1975). During the maturation of the rat adrenal medulla, catecholamines, their biosynthetic enzymes and storage vesicles, undergo a series of changes which are in part dependent upon the levels of neuronal input to the gland (Patrick & Kirshner, 1972; Slotkin, 1973a,b; 1975). The developing adrenal medulla does not respond to splanchnic stimulation in rats less than a week old, as shown by the inability of insulin to evoke reflex secretion of catecholamines (Slotkin, 1973b) and by the absence of trans-synaptic induction after reserpine administration (Bartolomé & Slotkin, 1976). This could result either from an inherent lack of ability of the adrenal to respond to nicotinic stimulation, or from failure of the splanchic nerve to deliver trans-synaptic signals to the adrenal. It is therefore essential to establish the pattern of development of nicotinic responses in the rat adrenal.

Previous studies indicate that maturing adrenergic systems respond to drugs differently from those of adults. Administration of morphine to pregnant and nursing rats produces long-term deficits in the offspring of adrenal catecholamines and catecholamine biosynthetic enzymes, a pattern unique to the immature animal (Anderson & Slotkin, 1975a). Reserpine or tetrabenazine administration to neonatal rats produces brain and adrenal catecholamine depletions that are more intense, more rapid in onset, and of longer duration than those produced in mature animals (Kulkarni & Shideman, 1966; Bartolomé & Slotkin, 1976). Furthermore, there appear to be periods in development during which exposure to drugs produces permanent alterations in adrenergic function (Bartolomé, Seidler, Anderson & Slotkin, 1976). In this study, the long-term effects of a single dose of nicotine administered to one day old rats have been examined in the developing adrenal medulla and central nervous system.

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## Methods

Pups from timed pregnant Sprague-Dawley rats (Zivic-Miller) were given 10 µl subcutaneous injections of nicotine 10 mg/kg or 0.9% w/v NaCl solution (saline). In studies to delineate the development of nicotinic responses, pups received single injections at various ages and were weighed and killed 3 h and 24 h later. To determine long-term effects, pups were given a single injection at one day of age, and the animals were weighed and killed at intervals of several days through young adulthood.

Adrenal glands were homogenized with a Polytron (Brinkmann Instruments) in 1.5–2.2 ml of 0.3 M sucrose containing 25 mM Tris (pH 7.4) and 10 µM iproniazid (irreversible monoamine oxidase inhibitor); 0.1 ml of homogenate was deproteinized with 1.9 ml of 3.5% perchloric acid and centrifuged at 26,000 *g* for 10 min; the supernatant was then analyzed for catecholamines by the trihydroxyindole method using an autoanalyzer (Merrills, 1963). An additional 0.5 ml of homogenate was added to 0.5 ml of water containing 2000 units/ml beef catalase, and then assayed in duplicate for dopamine $\beta$-hydroxylase by the method of Friedman & Kaufman (1965), using 10 µM [$^3$H]-tyramine as substrate and 0.5 mM parahydroxymercuribenzoate to inactivate endogenous inhibitors (Duch, Viveros & Kirshner, 1968). The remaining homogenate was centrifuged at 26,000 *g* for 10 min to sediment catecholamine storage vesicles, and 0.1 ml portions of the supernatant used for duplicate assays of tyrosine hydroxylase according to Waymire, Bjur & Weiner (1971), using 100 µM [$^{14}$C]-tyrosine as substrate. In order to obtain sufficient material for analysis before 11 days of age, gland pairs from 2–4 pups were pooled for each sample.

To examine central effects of neonatal nicotine administration, brains were weighed and homogenized in 9 volumes of Tris (pH 7.2) and duplicate 0.1 ml aliquots of homogenate assayed for tyrosine hydroxylase activity as described above, except that final concentrations of 0.1% Triton X-100 and 0.7 mM $CaCl_2$ were added to the assay to optimize activity. The rest of the homogenate was diluted with an equal volume of 10 mM Tris (pH 7.2), centrifuged at 26,000 *g* for 15 min and duplicate 0.9 ml portions of the supernatant used for assay of ornithine decarboxylase activity according to Anderson & Schanberg (1972), with 9.25 µM [$^{14}$C]-ornithine as substrate.

Data are reported as means ± standard errors, with levels of significance calculated by paired and unpaired *t* tests. Group means of control and experimental animals were paired by age over the time periods specified; degrees of freedom were calculated as the number of paired means minus one. This mode of pairing enables comparisons to be made of developmental patterns over the entire course of maturation or over multiple time periods as opposed to comparisons only of individual age points in the unpaired *t* test.

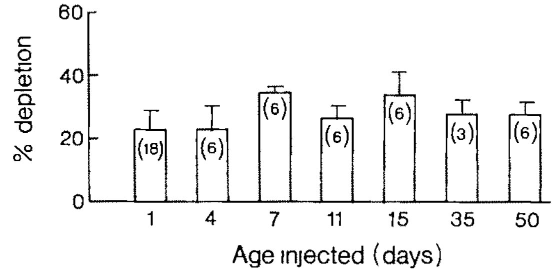

**Figure 1** Adrenal catecholamines 3 h after administration of nicotine (10 mg/kg, s.c.) to rats of various ages Data represent means of the number of determinations in parentheses, vertical lines show s.e. means, all points are significantly different from saline-treated controls by unpaired *t* test ($P < 0.05$ or better)

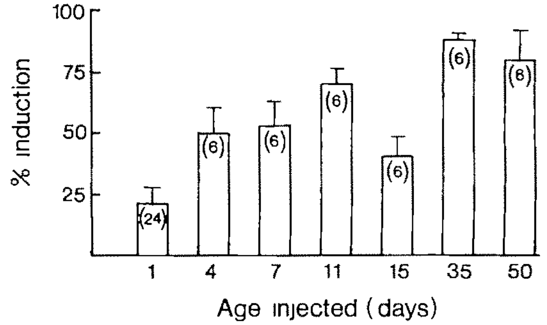

**Figure 2** Adrenal tyrosine hydroxylase activity 24 h after administration of nicotine (10 mg/kg, s c.) to rats of various ages. Data represent means of the number of determinations in parentheses, vertical lines show s.e. means; all points are significantly increased over saline-treated controls by unpaired *t* test ($P < 0.05$ or better).

Tyramine-[G-$^3$H] (5 Ci/mmol), L-tyrosine-[1-$^{14}$C] (55 mCi/mmol), and DL-ornithine-[1-$^{14}$C] (40 mCi/mmol) were purchased from New England Nuclear Corporation, and nicotine, iproniazid phosphate, beef catalase, and parahydroxymercuribenzoate from Sigma Chemical Company.

## Results

### *Development of nicotinic responses*

Three hours after nicotine administration to one day old rats, adrenal catecholamines were depleted by approximately 25% (Figure 1); nicotine given to rats at 4, 7, 11, 15, 35 or 50 days of age produced a similar

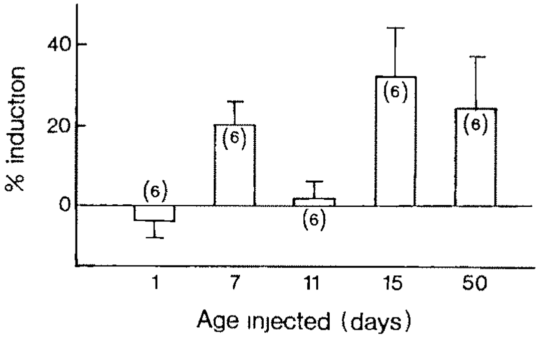

**Figure 3** Adrenal dopamine $\beta$-hydroxylase activity 24 h after administration of nicotine (10 mg/kg, s.c.) to rats of various ages. Data represent means of the number of determinations in parentheses, vertical lines show s.e. means; none of the differences is significant from controls by unpaired *t* test.

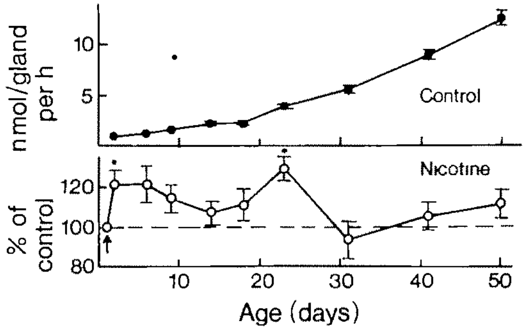

**Figure 5** Adrenal tyrosine hydroxylase activity in rats administered saline or nicotine (10 mg/kg, s.c.) at one day of age (at arrow). The first point after the arrow is at 24 h post-injection. Points and vertical lines represent means ± s.e. means of 11–24 determinations at each age; * significant differences from controls by unpaired *t* test ($P < 0.05$ or better).

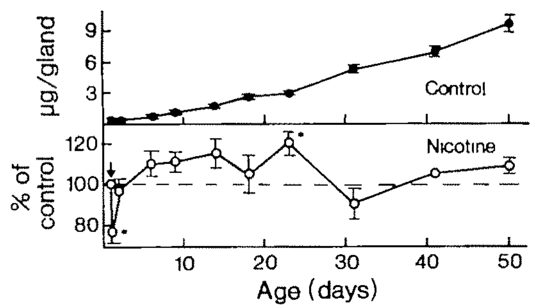

**Figure 4** Adrenal catecholamines in rats administered saline or nicotine (10 mg/kg, s.c.) at one day of age (at arrow). The first point after the arrow is at 3 h post-injection. Points and vertical lines represent means ± s.e. means of 11–24 determinations at each age; * significant differences from controls by unpaired *t* test ($P < 0.05$ or better).

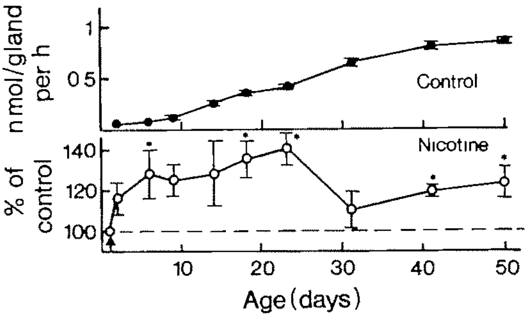

**Figure 6** Adrenal dopamine $\beta$-hydroxylase activity in rats administered saline or nicotine (10 mg/kg, s.c.) at one day of age (at arrow). The first point after the arrow is at 24 h post-injection. Points and vertical lines represent means ± s.e. means of 11–24 determinations at each age; * significant differences from controls by unpaired *t* test ($P < 0.05$ or better).

catecholamine depletion. In contrast, tyrosine hydroxylase induction 24 h after nicotine administration was higher in 50 day old rats (80% increase) than in one day old rats (21% increase, $P < 0.001$ *vs* 50-day induction, Figure 2). There appeared to be a significant correlation between age and ability to induce tyrosine hydroxylase ($r = 0.78$), except after injection at 15 days of age, where a smaller induction was noted than after 11 or 35 days.

At no age was significant induction of dopamine $\beta$-hydroxylase obtained 24 h after nicotine administration (Figure 3), and little or no alteration in body or brain weight or in brain tyrosine hydroxylase was seen either 3 or 24 h after nicotine (data not shown).

### *Long-term effects of neonatal administration*

Over the course of development, adrenal catecholamines in control rats increased from approximately 0.3 µg/gland to nearly 10 µg/gland in adulthood (Figure 4). After the acute (3 h) phase of adrenal catecholamine depletion caused by nicotine administration to one day old rats, levels recovered to normal by 2 days of age, and then tended toward elevation until 23 days of age ($P < 0.01$ by paired *t* test). From 31 to 50 days of age, adrenal catecholamines appeared to be normal (not significant by paired *t* test).

Adrenal tyrosine hydroxylase activity in control

**Table 1** Long-term effects of nicotine (10 mg/kg, s c.) administered at one day of age on body and brain weights, and on brain tyrosine hydroxylase activity

| *Age (days)* | *Body weight (g)* | | *Brain weight (g)* | | *Brain tyrosine hydroxylase (nmol/brain per h)* | |
|---|---|---|---|---|---|---|
| | *Control* | *Nicotine* | *Control* | *Nicotine* | *Control* | *Nicotine* |
| 2 | 9.5±0.2 (54) | 9.4±0.2 (54) | 0.361±0.004 (18) | 0.365±0.003 (18) | 4.2±0.4 (6) | 5.0±0.2 (6) |
| 6 | 15.9±0.7 (42) | 16.2±0.4 (42) | 0.686±0.007 (12) | 0.674±0.009 (12) | 7 8±0.4 (6) | 8.2±0.5 (6) |
| 9 | 24.0±1.0 (36) | 23.0±0.8 (36) | 0.988±0.013 (12) | 0.943±0.009 (11)† | 12 7±0.6 (6) | 12.3±0.3 (6) |
| 14 | 35.9±1.1 (12) | 35.1±1.6 (12) | 1.33±0.03 (6) | 1.29±0.02 (6) | 32.2±1.1 (6) | 32.5±1.2 (6) |
| 18 | 48.0±1 8 (18) | 45.6±2.6 (18) | 1.53±0.03 (6) | 1.42±0.01 (6)** | — | — |
| 23 | 67.7±4.6 (18) | 62.9±3 2 (18) | 1.64±0.05 (6) | 1.61±0.01 (6) | 61.3±1.6 (6) | 72.1±2.9 (6)* |
| 31 | 112±4 (12) | 111±5 (11) | 1.76±0.01 (6) | 1 62±0.02 (5)§ | 46.6±0 9 (6) | 41.4±0.2 (5)§ |
| 41 | 184±10 (12) | 182±9 (11) | 1.92±0.04 (6) | 1.83±0 02 (5) | 40.7±1.9 (6) | 44.8±1.1 (5) |
| 50 | 247±16 (14) | 239±14 (14) | 1.81±0.06 (6) | 1.79±0.03 (6) | 43.0±2.7 (6) | 44.2±2.0 (6) |

Data represent means ± s.e. means of the number of determinations in parentheses.
* $P<0.02$ vs. control by unpaired $t$ test; ** $P<0.01$; † $P<0.005$; § $P<0.001$.

**Table 2** Effects of nicotine (10 mg/kg, s c) administered at one day of age on brain ornithine decarboxylase activity

| *Age (days)* | *Brain ornithine decarboxylase (nmol/brain per h)* | |
|---|---|---|
| | *Control* | *Nicotine* |
| 2 | 0.64±0.04 (12) | 0 74±0.04 (12) |
| 6 | 0.55±0.04 (6) | 0.53±0 02 (6) |
| 9 | 0.56±0.03 (6) | 0.58±0.03 (6) |

Data represent means ± s.e. mean of the number of determinations in parentheses.

rats increased 13-fold over the course of development (Figure 5). Twenty four hours after neonatal nicotine administration, adrenal tyrosine hydroxylase activity was elevated 20%, and a tendency toward elevation persisted until 23 days of age ($P<0.02$ by paired $t$ test); from 31 to 50 days of age, no significant induction was noted. In contrast, induction of adrenal dopamine $\beta$-hydroxylase persisted after 23 days of age ($P<0.001$ until 23 days, $P<0.05$ 31–50 days by paired $t$ test) and was significant even into young adulthood (Figure 6).

Neonatal nicotine administration produced a minor retardation (5%) in developmental gains in body and brain weights ($P<0.05$ and $P<0.01$ by paired $t$ test, respectively); however, whole brain tyrosine hydroxylase activity appeared generally to be unaltered (not significant by paired $t$ test, Table 1).

No significant differences in the developmental fall-off of whole brain ornithine decarboxylase activity were seen at 2, 6, or 9 days of age, after neonatal nicotine administration (Table 2).

## Discussion

In adult animals, administration of agents which stimulate nicotinic receptors directly or which evoke central stimulation of the sympatho-adrenal axis (e.g. morphine, reserpine), causes release of adrenomedullary catecholamines (Schneider, 1969; Viveros, Arqueros, Connett & Kirshner, 1969; Anderson & Slotkin, 1975b; Slotkin & Seidler, 1975) and compensatory trans-synaptic induction of the catecholamine biosynthetic enzymes tyrosine hydroxylase (TH) and dopamine $\beta$-hydroxylase (DBH) (Thoenen, Mueller & Axelrod, 1969; Molinoff, Brimijoin, Weishilboum & Axelrod, 1970; Slotkin & Kirshner, 1973). In contrast, neonatal rats given reserpine show catecholamine depletion but not TH induction (Bartolomé & Slotkin, 1976), a phenomenon which could result from either absence of functional splanchnic innervation (Comline & Silver, 1966; Slotkin, 1973b) or from a lack of nicotinic receptors, or from a lessened inductive capability. In the present study, nicotine evoked an equivalent adrenomedullary response in adult and developing rats in terms of catecholamine depletion, but the degree of TH induction increased with age. These data show that functional nicotinic receptors are present in the neonatal adrenal gland before the establishment of functional innervation, which takes place at 7–10 days (Slotkin, 1973b; Bartolomé & Slotkin, 1976). However, there appears to be a difference in sensitivity between nicotinic receptor-mediated secretion of catecholamines and induction of TH, implying that secretion and induction are not coupled directly. This hypothesis is supported by observations in adult rats that low doses of nicotine or morphine can cause TH induction without an evident decrease in catecholamines (Slotkin & Seidler, 1975; Anderson & Slotkin, 1976), and that secretion without induction can occur in neonatal adrenals (Barolomé Slotkin, 1976). Since adrenal TH activity increased 13-fold over the first 50 days of development, nicotine-induced TH induction in the developing rat may be limited by the already high levels of transcription and/or translation (Thoenen, 1974). Alternatively, in the absence of innervation, receptor-mediated induction of TH inherently may be less effective than the induction seen in older animals; this latter hypothesis is supported in part by the observation that the rate of increase in TH is slower in denervated adult adrenals than in innervated glands during chronic nicotine administration (Seidler & Slotkin, 1976). However, immature adrenals are not simply the equivalent of denervated adult adrenals, since in the latter the acute catecholamine-secretory response to nicotine also is diminished (Seidler & Slotkin, 1976) while in neonates the TH induction is reduced without affecting catecholamine secretion.

It is of interest that, despite the general trend toward greater TH induction in older animals, a smaller TH induction was seen at 15 days than at 11 days ($P < 0.01$); similar results have been reported for reserpine (Bartolomé & Slotkin, 1976), suggesting that more subtle developmental changes may be superimposed upon the general pattern.

DBH activity was not induced 24 h after nicotine at any age tested. This probably results from the longer time period required for DBH induction, as well as exocytotic loss of soluble DBH (Patrick & Kirshner, 1971a; Slotkin & Seidler, 1975).

In long-term studies, a single dose of nicotine administered to 1-day old rats caused acute (3 h) catecholamine depletion, and subsequent persistent elevations of catecholamines, TH and DBH; however, while catecholamines and TH returned to normal by 31 days of age, DBH activity remained 20–30% above normal into young adulthood, suggesting a permanent increase in the number of storage vesicles (Viveros *et al.*, 1969; Slotkin, 1973b; Bartolomé *et al.*, 1976). Three hypotheses could explain this phenomenon: first, the chronically increased DBH could be due to trans-synaptic induction *via* permanently increased sympathoadrenal outflow. However, one would then also expect a permanent induction of TH to a larger extent than for DBH; the smaller change in TH indicates that trans-synaptic induction alone cannot account for the magnitude of change in DBH. Second, since nicotine causes release of adrenocorticotrophic hormone (ACTH), as well as direct secretion of adrenocortical steroids (Kershbaum, Pappajohn, Belley, Hirabayashi & Shafiiha, 1968; Suzuki, Ikeda, Narita, Shibata, Waki & Egashira, 1973; Rubin & Warner, 1975), a primary effect upon the pituitary-adrenal axis could cause long-term corticoid-mediated changes in TH and DBH; this is unlikely because ACTH apparently increases adrenal TH and DBH only in hypophysectomized animals (Mueller, Thoenen, Axelrod, 1970; Weinshilboum & Axelrod, 1970), and because the effect is usually greater for TH than for DBH (in contrast to the present findings). Further to rule out corticoids as a primary mediator of adrenomedullary developmental alterations, the effect of nicotine on brain ornithine decarboxylase (ODC) was evaluated. Anderson & Schanberg (1975) have shown that corticoids produce a marked lag in the developmental fall-off of brain ODC. In the present study, a normal ODC pattern was seen, suggesting that adrenocortical secretion is unaffected.

The third hypothesis which could explain long-term elevations in adrenal DBH after neonatal nicotine administration is that the adrenal medulla itself is altered. In this regard, in addition to trans-synaptic or corticoid-mediated regulation of DBH, another mechanism appears to exist which is mediated directly in the adrenal medulla and which is unique to DBH. In adult rats, this mechanism can be unmasked by denervation of adrenals, followed by treatment with reserpine (Patrick & Kirshner, 1971b), chlorisondamine (Slotkin, Seidler, Lau, Bartolomé & Schanberg, 1976), or morphine (Anderson & Slotkin, 1976); while induction of DBH is seen under these conditions, no TH induction occurs. Thus, in addition to trans-synaptic effects, a long-term developmental change in DBH regulation in the adrenal medulla itself may be responsible for the persistent elevation in activity of that enzyme.

In conclusion, adrenomedullary nicotinic receptors are present in the neonatal rat before development of functional splanchnic innervation, but the ability to induce TH is lower than that in adults. A single dose of nicotine administered at one day of age can produce long-term changes in control of catecholamine biosynthetic enzymes.

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# THE EFFECT OF $\Delta^9$-TETRAHYDROCANNABINOL ON BODY TEMPERATURE AND BRAIN AMINE CONCENTRATIONS IN THE RAT AT DIFFERENT AMBIENT TEMPERATURES

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1 Rats were injected intravenously with 2 mg/kg (–)-*trans*-$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) at ambient temperatures of 4°, 21°, 31° and 37°C.

2 The general behaviour exhibited by rats treated with $\Delta^9$-THC was similar at all four ambient temperatures.

3 Body temperatures were recorded continuously before and after drug administration. At 4° and 21°C, $\Delta^9$-THC caused hypothermia whereas no change in body temperature occurred at 31° and 37°C.

4 The concentrations in the whole brain of noradrenaline (NA), dopamine, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) were determined spectrophotofluorimetrically 1 h after drug administration. At 4°C $\Delta^9$-THC caused an increase of 5-HT, at 21°C an increase of 5-HIAA, at 31°C an increase of 5-HIAA and a decrease of NA, and at 37°C an increase of 5-HT and 5-HIAA.

5 At all ambient temperatures, $\Delta^9$-THC increased the brain levels of 5-HT and/or 5-HIAA. A correlation between the $\Delta^9$-THC-induced hypothermic response and the possible alteration of brain 5-HT metabolism cannot be excluded.

## Introduction

It is well documented that the administration of (–)-*trans*-$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) to laboratory animals results in hypothermia (Holtzman, Lovell, Jaffe & Freedman, 1969; Lomax, 1971; Sofia, 1972). The investigations of Feldberg & Myers (1963; 1964; 1965) provide the basis for the hypothesis that catecholamines and 5-hydroxytryptamine (5-HT) are involved in the central mechanism of temperature control. Holtzman *et al.* (1969) suggested that the hypothermic response in mice induced by $\Delta^9$-THC is correlated to changes in the brain levels of noradrenaline (NA) and 5-HT. Other workers have found that $\Delta^9$-THC or cannabis extracts cause brain NA concentrations to fall (Schildkraut & Efron, 1971), to rise (Constantinidis & Miras, 1971) or to remain unchanged (Maître, Staehelin & Bein, 1970; Mazurkiewicz-Kwilecki & Filczewski, 1973), whereas concentrations of 5-HT have been reported to increase (Schildkraut & Efron, 1971; Sofia, Dixit & Barry, 1971) or to remain unchanged (Gallager, Sanders-Bush & Sulser, 1972; Yagiela, McCarthy & Gibb, 1974).

In the light of these conflicting reports, it was decided to investigate the effect of $\Delta^9$-THC on the body temperature of rats maintained at different ambient temperatures, and at the same time to examine whether a correlation exists between changes in body temperature and changes in brain monoamine concentrations. At different ambient temperatures, the $\Delta^9$-THC-induced hypothermic response is modified (Haavik & Hardman, 1973) as is the dynamic state of brain monoamines (Reid, Volicer, Smookler, Beaven & Brodie, 1968).

## Methods

### *Animals*

Male albino Wistar rats weighing between 200 and 300 g were housed in an animal room at an ambient temperature of 21 ± 2°C and a 12 h light-dark cycle. Food and water were available *ad libitum*. For intravenous injection, a permanent polyethylene (PE 10) catheter was inserted into the external jugular vein under amylobarbitone sodium plus methohexitone sodium (i.p.) anaesthesia. Before surgery, animals were housed in group cages, but for the 48 h recovery period and during the experimental period, they were kept in individual cages. Rats were used either for body temperature studies or brain amine determinations. All experiments were completed before midday.

### Studies at different ambient temperatures

Four ambient temperatures were used in these studies, namely 4 ± 1°, 21 ± 2°, 31 ± 1° and 37° to 39°C. At temperatures other than 21°C the animals were allowed a 3 h acclimatization period before the injection of $\Delta^9$-THC or its vehicle.

### Preparation of $\Delta^9$-THC

A dispersion of $\Delta^9$-THC in saline (0.9% w/v NaCl solution) using polyvinylpyrrolidone (PVP) following the method of Fenimore & Loy (1971) was used. $\Delta^9$-THC (2 mg/kg) or PVP (40 mg/kg) was injected intravenously in a volume of 1 ml/kg body weight.

### Body temperature studies

The body temperature of individually caged, unrestrained rats was recorded continuously for 3 h by means of a thermistor probe inserted 60–70 mm into the rectum 1 h before the administration of $\Delta^9$-THC or PVP. Three thin pieces of adhesive tape held the probe in position relative to the tail. Temperatures were recorded continuously with either an Offner type R or a Riken Denshi pen recorder.

### Brain amine determinations

One hour after the intravenous injection of $\Delta^9$-THC 2 mg/kg or PVP 40 mg/kg, rats were killed by decapitation. Their brains were rapidly removed, blotted free from blood, frozen in liquid $N_2$ and stored at −20°C. For amine determinations, the frozen brains were weighed and, after thawing in a solution of 0.4 M perchloric acid containing 0.2% disodium edetate, homogenized in a motor driven, all glass homogenizer. The amines dopamine, NA and 5-HT were extracted from the homogenate using BIO-REX 70 (BIO-RAD Laboratories) according to the column procedure of Barchas, Erdelyi & Angwin (1972). 5-Hydroxyindoleacetic acid (5-HIAA) was further extracted from the first effluent off the column into *n*-butyl acetate made acidic by addition of 100 µl 3 M HCl. After removal of the aqueous phase, 5 ml 0.5 M phosphate buffer pH 7.0 was added to the organic phase and 5-HIAA was re-extracted into the buffered phase. The amines NA, dopamine and 5-HT were then eluted from the column with 5 ml 0.5 M acetic acid.

The extracted amines were then assayed spectrophotofluorimetrically as follows: dopamine, according to the method of Carlsson & Waldeck (1958); NA, according to the method of Weil-Malherbe (1971) and 5-HT and 5-HIAA according to the method of Lovenberg & Engelman (1971). The fluorescence developed was determined with an Aminco-Bowman spectrophotofluorimeter. The concentrations of brain amines are expressed in terms of the free base or acid.

### Analysis of results

In the body temperature studies, results are expressed in 'Thermic Index' values as defined by Jori, Paglialunga & Garattini (1967). The Thermic Index is a measurement of the cumulative change in body temperature from the original which is calculated 5, 10, 15, 30, 45, 60, 75, 90, 105 and 120 min after the injection of $\Delta^9$-THC or its vehicle. For testing the statistical significance of differences between means, Student's *t*-test was used (with the aid of a Cyber 74 computer).

### Drugs

The following drugs and chemicals were used: amylobarbitone sodium (Amytal, Eli Lilly); dopamine hydrochloride (3-hydroxytyramine, Sigma); 5-hydroxyindoleacetic acid cyclohexyl ammonium salt (Sigma); 5-hydroxytryptamine creatinine sulphate (Koch-Light); methohexitone sodium (Brietal Sodium, Eli Lilly); (−)-noradrenaline hydrochloride (Levarterenol, Sigma); polyvinylpyrrolidone (Kollidon 25, BASF); (−)-*trans*-$\Delta^9$-THC (Batch SSC 75814, NIMH). All reagents used for extraction and assay were of analytical grade.

## Results

### General behaviour

The behaviour of the rats was observed for a 2 h period following the intravenous injection of $\Delta^9$-THC 2 mg/kg. After about 3 min the animals started an exploratory type of behaviour manifested as periodic walking around the cage and digging in the sawdust with their noses. This was accompanied by spontaneous jumping or 'jumping fits'. This period of abnormal motor activity, which lasted for about 20 min, appeared to be superimposed on a state of depression which became evident 10 min after the injection of $\Delta^9$-THC. The depression was manifested as catatonia, depressed respiration and splayed rear legs. There appeared to be a decreased threshold to tactile stimuli because the rats squealed when held or touched. Catatonia lasted for about 1 hour. For the following hour general sedation prevailed. This type of behaviour occurred at each of the four ambient temperatures.

**Table 1** Effect of four different ambient temperatures on the body temperatures of individually caged, untreated rats

| *Ambient temperature (°C)* | *Body temperature* (°C ± s.e. mean)* | *Level of significance‡ (P value)* 4°C | 21°C | 31°C | 37°C | *Body weight† (g ± s.e. mean)* |
|---|---|---|---|---|---|---|
| 4 | 37.42 ± 0.19 | — | 0.212 | 0.002 | 0.000 | 256.5 ± 8.1 (15) |
| 21 | 37.72 ± 0.15 | 0.212 | — | 0.008 | 0.000 | 260.4 ± 5.5 (21) |
| 31 | 38.49 ± 0.23 | 0.002 | 0.008 | — | 0.001 | 255.8 ± 9.1 (11) |
| 37 | 39.56 ± 0.17 | 0.000 | 0.000 | < 0.001 | — | 246.9 ± 7.9 (15) |

The figures in parentheses are the number of rats in each group. † The mean body weights of each group were not significantly different from each other.

* Body temperatures were recorded at 3 h after rats were placed in the different ambient temperature environments.

‡ *P* values were calculated by comparison of the body temperatures of the groups of rats at the different ambient temperatures.

**Table 2** Effect of intravenous injections of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) 2 mg/kg and polyvinylpyrrolidone (PVP) 40 mg/kg on the Thermic Index values of individually caged rats at different ambient temperatures

| *Ambient temperature (°C)* | *Thermic Index ± s.e. mean* PVP | $\Delta^9$-THC | *Body weight† ± s.e. mean* PVP | $\Delta^9$-THC |
|---|---|---|---|---|
| 4 | +0.96 ± 2.22 (6) | −15.41 ± 1.86 (9)* | 260.3 ± 14.2 (6) | 254.0 ± 10.3 (9) |
| 21 | −1.80 ± 0.94 (10) | −11.66 ± 1.65 (11)* | 259.0 ± 9.9 (10) | 261.6 ± 5.8 (11) |
| 31 | +0.17 ± 2.83 (5) | −2.97 ± 2.11 (6) | 256.2 ± 14.3 (5) | 255.5 ± 13.1 (6) |
| 37 | −0.20 ± 0.58 (6) | +1.49 ± 0.54 (9) | 254.7 ± 12.7 (6) | 247.8 ± 10.6 (9) |

Rats were injected either with $\Delta^9$-THC or PVP 3 h after being placed in a specific ambient temperature where they remained for at least a further 2 hours. Figures in parentheses indicate the number of animals in each group. † The mean body weights were not significantly different from each other.
The Thermic Index is a measurement of the cumulative change in body temperature from the original calculated at 5, 10, 15, 30, 45, 60, 75, 90, 105 and 120 min after drug administration.
* $P < 0.001$, compared with PVP.

**Table 3** Mean body weight of rats receiving either polyvinylpyrrolidone (PVP) 40 mg/kg or $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) 2 mg/kg

| *Ambient temperature (°C)* | *Body weight (g ± s.e. mean)** PVP | $\Delta^9$-THC |
|---|---|---|
| 4 | 259.0 ± 6.3 (24) | 253.2 ± 4.1 (47) |
| 21 | 250.9 ± 6.6 (18) | 255.9 ± 4.8 (36) |
| 31 | 254.5 ± 8.2 (12) | 260.3 ± 5.4 (24) |
| 37 | 250.5 ± 7.6 (13) | 248.8 ± 5.2 (23) |

Brains from these animals were used in the determination of brain amine concentrations at different ambient temperatures. Figures in parentheses indicate the number of rats in each group. * The mean body weights of each group were not significantly different from each other.

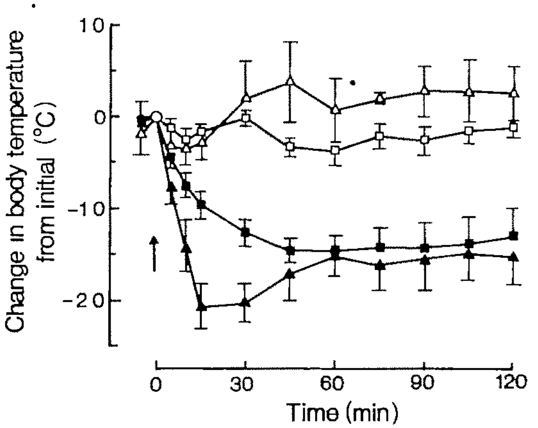

**Figure 1** Effect of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) 2 mg/kg injected intravenously, on the body temperature of rats kept at ambient temperatures of 4° (▲) and 21 °C (■). For comparison, the effect of an injection of polyvinylpyrrolidone (PVP, 40 mg/kg) on the body temperature of rats kept at 4° (△) and 21 °C (□) is also shown. Each point is the mean of results from at least 6 rats. Vertical lines show s.e. means. The body temperatures were recorded for 1 h before, and 2 h after the injections at time 0 as indicated by the arrow.

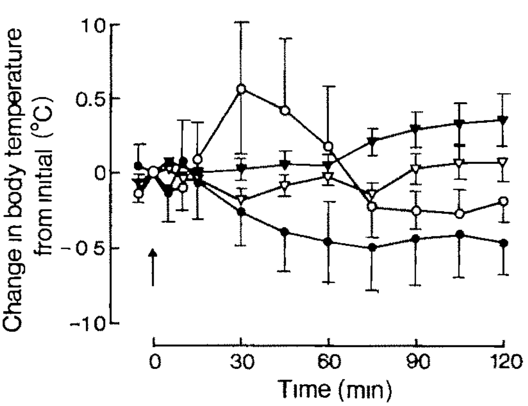

**Figure 2** Effect of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) 2 mg/kg, injected intravenously, on the body temperature of rats kept at ambient temperatures of 31° (●) and 37 °C (▼). For comparison the effect of an injection of polyvinylpyrrolidone (PVP, 40 mg/kg) on the body temperature of rats kept at 31° (○) and 37 °C (▽) is also shown. Each point is the mean of results from at least 5 rats. Vertical lines show s.e. means. The body temperatures were recorded for 1 h before, and 2 h after the injections at time 0 as indicated by the arrow.

### *Effect of different ambient temperatures on the body temperature of untreated rats*

Table 1 shows the mean body temperatures of untreated rats as recorded 3 h after the animals were placed at ambient temperatures of 4°, 21°, 31° or 37°C. The continuous recording of the rectal temperature was started 2 h after introducing the rats to the various ambient temperatures. The body temperatures of rats at 37°C were significantly higher than those of rats at 4°, 21° and 31°C ($P < 0.001$). Rats kept at 31°C had a significantly higher body temperature than those kept at 4° and 21°C ($P < 0.01$). There was no significant difference between the body temperatures of rats at 4° and 21°C.

The mean body weights of rats in each group in all experiments have been included in Tables 1–3 to show that, although animals having a large weight range were used, there was no significant difference in the mean body weights between the groups.

### *Effect of ambient temperature on the body temperature of rats injected with $\Delta^9$-THC*

The temperature of rats injected with $\Delta^9$-THC 2 mg/kg and kept at ambient temperatures of 4° and 21°C was monitored for 2 h following the injection (see Figure 1). At both ambient temperatures $\Delta^9$-THC significantly decreased body temperature compared to PVP-treated controls. At 21°C the maximum change in body temperature was reached approximately 1 h after administration, at 4°C the maximum hypothermic effect (–2.1 °C) occurred 15 min after the administration of $\Delta^9$-THC. At both ambient temperatures, 6 to 8 h elapsed before body temperature returned to pre-injection values.

Figure 2 shows the body temperature of rats kept at 31° and 37°C during the 2 h period following administration of $\Delta^9$-THC 2 mg/kg. At these elevated ambient temperatures $\Delta^9$-THC did not decrease the body temperatures when compared with those of PVP-treated controls. At 37°C there even occurred a significant increase ($P < 0.02$) 75 min after the injection of $\Delta^9$-THC.

To enable a statistical comparison of the effects of $\Delta^9$-THC 2 mg/kg and PVP 40 mg/kg on the body temperature of rats placed at the four ambient temperatures, the Thermic Index values were calculated from the temperature measured at 5, 10, 15, 30, 45, 60, 75, 90, 105 and 120 min following drug administration (see Table 2). At the four ambient temperatures the Thermic Index values of rats treated with PVP were not significantly different from one another. The Thermic Index values of rats treated with $\Delta^9$-THC at 4° and 21°C were not significantly different from one another; however, as the ambient temperature was increased from 21° to 31°C and from 31° to 37°C, the Thermic Index values of rats treated with $\Delta^9$-THC increased significantly ($P < 0.05$). The Thermic Index values obtained from rats treated with $\Delta^9$-THC compared to those of PVP-treated rats were significantly lower at 4° and 21°C ($P < 0.001$), whereas at 31° and 37°C there was no significant difference between the two treatments.

### *Effect of ambient temperature and $\Delta^9$-THC on the concentration of amines in whole brain*

The NA and dopamine concentrations in whole brain are tabulated in Table 4. No significant differences occurred between the concentrations of NA in the brains of PVP-treated rats at each of the four ambient temperatures. There was a small reduction (−9%) in brain dopamine in PVP-treated animals at 31°C when compared to that at 21°C ($P < 0.05$). At the four ambient temperatures, $\Delta^9$-THC did not alter the brain concentrations of NA and dopamine, except for a small (−8%) but statistically significant ($P < 0.02$) decrease in the NA concentration at 31°C.

The concentrations of 5-HT and 5-HIAA in the whole brain are shown in Table 5. The 5-HT concentration in PVP-treated rats kept at 37°C was significantly greater (+9% and +11%) than that of PVP-treated animals at 4° and 21°C ($P < 0.05$). The 5-HIAA concentration in PVP-treated rats kept at 37°C was also significantly higher (+35%, 38% and 15%) than that of PVP-treated rats kept at 4°, 21° and 31°C ($P < 0.02$). These values obtained at 31°C were also significantly higher (+20%, +18%) than those at 4° and 21°C ($P < 0.001$). Administration of $\Delta^9$-THC caused a significant increase in the brain content of 5-HT by 16% and 10% in rats kept at 4° and 37°C when compared with that of PVP-treated control animals kept at the same temperatures ($P < 0.01$). At an ambient temperature of 21°C, $\Delta^9$-THC increased the brain content of 5-HIAA by 32% relative to PVP-treated rats ($P < 0.0001$). In rats kept at 31°C, there was also an increase in the brain content of 5-HIAA (+18%, $P < 0.005$) compared to that of PVP-treated controls. In the rats kept at 37°C, the increase amounted to +15% ($P < 0.05$).

## Discussion

At ambient temperatures of 4°, 21°, 31° and 37°C the general behaviour of rats following the intravenous administration of $\Delta^9$-THC 2 mg/kg in this

**Table 4** Concentrations of noradrenaline and dopamine in whole brain samples of rats 1 h after intravenous injection of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) 2 mg/kg or polyvinylpyrrolidone (PVP) 40 mg/kg at different ambient temperatures

| *Ambient temperature (°C)* | *Noradrenaline (µg/g ± s.e. mean)* | | *Dopamine (µg/g ± s.e. mean)* | |
|---|---|---|---|---|
| | *PVP* | *$\Delta^9$-THC* | *PVP* | *$\Delta^9$-THC* |
| 4 | 0.413 ± 0.028 (22) | 0.457 ± 0.020 (42) | 0.910 ± 0.027 (23) | 0.958 ± 0.022 (47) |
| 21 | 0.434 ± 0.024 (18) | 0.422 ± 0.022 (35) | 0.911 ± 0.023 (18) | 0.928 ± 0.022 (36) |
| 31 | 0.446 ± 0.008 (12) | 0.408 ± 0.006 (24)* | 0.831 ± 0.026 (12) | 0.814 ± 0.029 (24) |
| 37 | 0.462 ± 0.012 (13) | 0.460 ± 0.005 (23) | 0.858 ± 0.028 (13) | 0.838 ± 0.025 (23) |

Figures in parentheses indicate the number of animals in each group Significance of difference from corresponding PVP controls: * $P < 0.002$.

**Table 5** Concentrations of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in whole brain samples of rats 1 h after intravenous injection of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) 2 mg/kg or polyvinylpyrrolidone (PVP) 40 mg/kg at different ambient temperatures

| *Ambient temperatures (°C)* | *5-HT (µg/g ± s.e. mean)* | | *5-HIAA (µg/g ± s.e. mean)* | |
|---|---|---|---|---|
| | *PVP* | *$\Delta^9$-THC* | *PVP* | *$\Delta^9$-THC* |
| 4 | 0.452 ± 0.010 (22) | 0.525 ± 0.013 (47)‡ | 0.907 ± 0.026 (24) | 0.964 ± 0.034 (45) |
| 21 | 0.443 ± 0.010 (16) | 0.451 ± 0.009 (36) | 0.890 ± 0.033 (12) | 1.178 ± 0.034 (23)‡ |
| 31 | 0.477 ± 0.019 (12) | 0.492 ± 0.020 (24) | 1.070 ± 0.027 (11) | 1.262 ± 0.034 (24)‡ |
| 37 | 0.491 ± 0.016 (13) | 0.545 ± 0.011 (23)† | 1.226 ± 0.046 (13) | 1.418 ± 0.061 (23)* |

Figures in parentheses indicate the number of animals in each group.
Significance of difference from corresponding PVP controls: * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.002$.

study was qualitatively similar to that observed by Gallager *et al.* (1972) and Paton & Pertwee (1973). The jumping fits observed in about half the animals within the first 15 min after drug administration have also been reported by Schildkraut & Efron (1971) and Goldman, Dagirmanjian, Drew & Murphy (1975). The latter authors propose that this jumping behaviour may involve tryptaminergic mechanisms. Our results are in agreement with this suggestion since changes in the brain concentrations of 5-HT and/or 5-HIAA occurred at all four ambient temperatures. This study also confirms that, in laboratory animals, administration of $\Delta^9$-THC causes a decrease in body temperature (Holtzman *et al.*, 1969; Lomax, 1971; Sofia, 1972). The magnitude of the hypothermic response to $\Delta^9$-THC 2 mg/kg calculated as the Thermic Index, is dependent on ambient temperature. As the ambient temperature increases from 4° to 37°C there is a corresponding increase in the Thermic Index of $\Delta^9$-THC treated rats; at ambient temperatures of 4° and 21°C, $\Delta^9$-THC produces hypothermia, whereas at ambient temperatures of 31° and 37°C, no significant hypothermia is observed. These results are in agreement with those of Haavick & Hardman (1973) who observed a progressive decrease of the hypothermic response of mice to $\Delta^9$-THC at ambient temperatures between 10°–35°C. The rate of change of body temperature after $\Delta^9$-THC injection was also dependent on the ambient temperature. At an ambient temperature of 4°C the lowest values were already reached 15 min after the drug injection, at 21°C only after about 40 minutes. After 45 min there was no difference in the body temperatures of drug-treated animals kept at 4° or 21°C. The body temperatures of both groups of rats remained significantly lower than those of PVP-injected control animals kept at these ambient temperatures. These results indicate that in the rat $\Delta^9$-THC produces hypothermia and not poikilothermia as has been observed in dogs after administration of the synthetic tetrahydrocannabinol derivative, dimethyl heptyl pyran (Hardman, Domino & Seevers, 1971).

The differences in body temperature of untreated rats observed 3 h after exposure to ambient temperatures of 4°, 21°, 31° or 37°C (see Table 1) are similar to those reported by Ingenito & Bonnycastle (1967) and Simmonds (1969). The hyperthermia seen in control rats at 31° and 37°C was not associated with alterations of the brain concentrations of NA or dopamine as has also been reported by Ingenito & Bonnycastle (1967) and Reid *et al.* (1968). Simmonds (1969) found that alterations of ambient temperature resulted in an increased turnover of NA, although the concentrations of NA in the hypothalamus remained unaltered. The control rats studied in the present experiments showed significantly higher brain concentrations of 5-HIAA when they were kept at 31° and 37°C than at 4° and 21°C. The brain concentrations of 5-HT in control rats only increased as the ambient temperature increased to 37°C. These results are in agreement with those of Reid *et al.* (1968) and Tagliamonte, Tagliamonte, Perez-Cruet, Stern & Gessa (1971) but differ from those of Ingenito & Bonnycastle (1967).

The effect of $\Delta^9$-THC on rat brain monoamines appeared to be associated with changes in the brain content of 5-HT and 5-HIAA. When compared with PVP-treated rats, the $\Delta^9$-THC treated rats showed at 4°C an increase in 5-HT, at 21°C an increase in 5-HIAA, at 31°C an increase in 5-HIAA (and a decrease in NA) and at 37°C an increase in both 5-HT and 5-HIAA. The small fall in the NA level at 31°C agrees with the observations of Holtzman *et al.* (1969) and Schildkraut & Efron (1971). The observation that $\Delta^9$-THC did not alter the brain concentration of dopamine at any ambient temperature is consistent with the findings of Maître *et al.* (1970), who showed that, while rat brain NA and dopamine contents were unaffected by $\Delta^9$-THC, there was an increase in brain catecholamine turnover.

In preliminary experiments with pargyline we observed that $\Delta^9$-THC does not block acid transport from the central nervous system. Consequently, at ambient temperatures of 21°, 31° and 37°C, the rises in 5-HIAA concentrations may indicate an alteration in 5-HT metabolism. An increase in brain 5-HT content after $\Delta^9$-THC was also reported by Sofia *et al.* (1971) but not by Gallager *et al.* (1972). Since changes in ambient temperature can alter monoamine metabolism (Reid *et al.*, 1968; Simmonds, 1969; 1970), it appears reasonable to suggest that $\Delta^9$-THC has altered the 5-HT metabolism at all four ambient temperatures used.

In conclusion, the results of the present investigation demonstrate that the $\Delta^9$-THC-induced hypothermia is dependent on ambient temperature. Although the apparent alteration in 5-HT metabolism at all ambient temperatures may not be linked with the hypothermic responses, such a correlation cannot be excluded.

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# INTRARENAL STIMULATION OF RENIN SECRETION BY FRUSEMIDE IN THE ISOLATED KIDNEY OF THE RAT

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1 Intrarenal infusion of frusemide markedly stimulated renin secretion in the isolated perfused kidney of the rat.

2 Renin values increased from 24 ± 6 to 195 ± 34 units of secretion rate (renin concentration (nmol angiotensin I/h per litre) × flow rate (ml/min)) following administration of frusemide for 8 min, compared with corresponding control values of 13 ± 2 ($P > 0.05$) and 47 ± 18 ($P < 0.001$). This stimulatory effect was also observed when urine flow was interrupted by ligation of the ureters.

3 The changes in renal perfusion pressure and perfusate flow rate were not significantly different from the control values.

4 These findings indicate the existence of an intrarenal site of action for frusemide on renin secretion.

5 Since frusemide did not appear to alter perfusion pressure or flow rate, and was effective when urine flow was abolished, a dominant role for a vascular or macula densa receptor mechanism seems unlikely. A direct effect of frusemide on the renin secreting cell is therefore suggested.

## Introduction

The administration of potent natriuretic agents such as frusemide leads to marked stimulation of renin release (Fraser, James, Brown, Isaac, Lever & Robertson, 1965; Rosenthal, Boucher, Nowaczynski & Genest, 1967; Vander & Luciano, 1967). The simplest explanation is that renin secretion is stimulated by the reduction in renal perfusion pressure (Skinner, McCubbin & Page, 1964) or the secondary release of catecholamines (Gordon, Küchel, Liddle & Island, 1967; Winer, Chokshi, Yoon & Freedman, 1969) subsequent to an acute fall in plasma volume. However, studies in dogs (Vander & Carlson, 1969) and rabbits (Meyer, Menard, Papanicolaou, Alexandre, Devaux & Milliez, 1968) have demonstrated that the stimulation of renin secretion by frusemide persists even when changes in body sodium and water balance are prevented. This is supported by a recent study in man showing that the rapid increase in plasma renin activity following intravenous frusemide did not correlate with plasma volume changes (Hesse, Nielsen & Lund-Jacobson, 1975).

Although these observations suggest an intrarenal site of action of frusemide on renin release, extrarenal factors cannot be excluded by the experimental conditions employed. In the study described here the isolated perfused kidney of the rat (Vandongen, Peart & Boyd, 1973) has been used to investigate a possible intrarenal site of action of frusemide on renin release and to relate this to changes in renal perfusion pressure and flow rate.

## Methods

### *Kidney perfusion*

Male wistar rats (150–250 g) maintained on similar diets were anaesthetized with sodium pentobarbitone (0.1 mg/g by intraperitoneal injection) and given heparin (50–100 units intravenously). The left kidney was cannulated and perfused with Krebs–Ringer saline by means of a pulsatile flow inducer pump (Watson Marlow MHRE 1000) as previously described (Vandongen *et al.*, 1973) except that in most experiments the ureter was occluded by ligature before starting the perfusion. Perfusion pressure was monitored continuously by a pressure transducer (Bell and Howell) and recorder (Devices MX2). Approximately 5 min after starting the perfusion, which was designated 0 min, a timed collection of effluent perfusate was obtained for determination of renin concentration. At 1 min the infusion of frusemide (Lasix, supplied by Hoechst Australia, Pty. Ltd.) diluted in distilled water to the required concentration, or distilled water only (control study), was started at the rate of 0.04 ml/min and this was continued for the duration of the experiment. Further collections of perfusate were obtained at 5 min and 9 min (4 and 8

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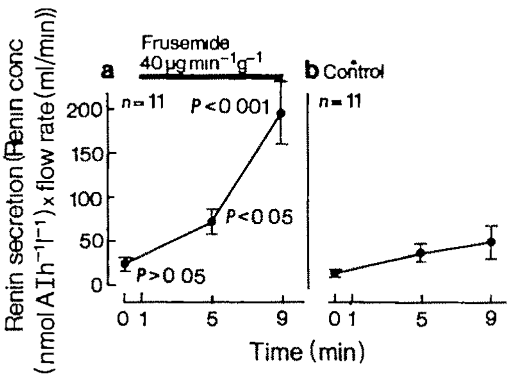

**Figure 1** The effect of frusemide, infused from 1 to 9 min, on renin secretion rate (a). The control study without frusemide infusion is shown in (b). All values are means of 11 experiments. Vertical lines show s e. means. $P$ values refer to significance of difference between frusemide and corresponding control values (Student's nonpaired $t$ test). AI = angiotensin I.

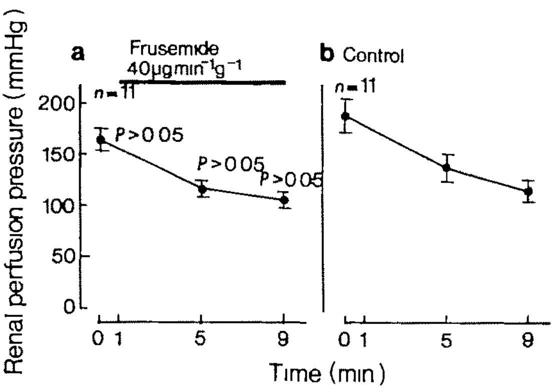

**Figure 2** The effect of frusemide, infused from 1 to 9 min, on renal perfusion pressure (a) The control study without frusemide infusion is shown in (b). All values are means of 11 experiments. $P$ values refer to significance of difference between frusemide and corresponding control values (Student's nonpaired $t$ test).

min respectively after the start of frusemide or distilled water infusion). The doses of frusemide are expressed per g wet kidney weight (non-perfused kidney). Control and frusemide experiments were performed alternately.

### *Renin assay*

Timed collections of perfusate were treated according to the method of Skinner (1967) for plasma renin activity. After incubation for 6 h with nephrectomized rat plasma as renin substrate source (treated as sheep substrate by the method of Skinner) further reaction was terminated by heating at 85°C for 5 min, and the samples assayed without extraction for angiotensin I by radioimmunoassay (Boyd, Adamson, Fitz & Peart, 1969). Renin concentration is expressed in nmol equivalents of 1-Asp-5-Ile-angiotensin I generated per h per litre of perfusate and converted into secretion rate by multiplying by the flow rate (ml/min). All values shown are mean values ± s.e. mean. Statistical analysis was carried out using non-paired Student's $t$ test and 2-tailed probability tables. All samples from any one experiment were processed and assayed at the same time.

## Results

Figure 1 compares the response in renin secretion rate to frusemide (40 µg $\mathrm{min}^{-1}$ $\mathrm{g}^{-1}$), after infusion for 4 and 8 min in 11 experiments, with that observed in 11 control experiments conducted over the same period of time. Following the introduction of frusemide at 1 min, renin secretion (as defined in the Methods section) increased from 24 ± 6 at 0 min to 72 ± 13 at 5 min and to 195 ± 34 at 9 minutes. In 2 experiments where the ureter was not occluded, renin secretion increased from 24 and 15 at 0 min to 355 and 246, respectively, at 9 minutes. In the control studies the rise in renin secretion observed, 13 ± 2 at 0 min to 35 ± 10 at 5 min and 47 ± 18 at 9 min, was similar to that reported in previous studies with this preparation (Vandongen & Greenwood, 1975b) but was significantly lower than during the infusion of frusemide. When the individual changes are expressed as a percentage of the 0 min value, the mean increase at 9 min is 283% in the control and 927% in the frusemide group ($P < 0.05$).

The response to frusemide is at least partly determined by the basal secretion rate at 0 min since a significant inverse relationship was found between this and the percentage increase at 9 min ($r = -0.66$, $P < 0.05$).

The changes in renal perfusion pressure during the infusion of frusemide and in the corresponding control study are shown in Figure 2. Although the initial and subsequent pressures were somewhat higher in the control group, these values and the actual percentage fall from 0 min to 9 min (36 ± 3% for the frusemide and 35 ± 4% for the control group) were not significantly different. During this progressive fall in renal perfusion pressure flow rates increased marginally, 7.4 ± 0.4 at 0 min to 8.3 ± 0.4 ml/min at 9 min in the frusemide and 8.4 ± 0.7 to 9.5 ± 0.5 ml/min in the control group, there being no significant differences between the two groups ($P > 0.05$).

This finding indicates that renal autoregulatory function was preserved.

## Discussion

Previous observations that intravenous frusemide increased plasma renin levels in animals even when urinary losses were replaced (Bailie, Davis & Loutzenhiser, 1973) have been cited in support of an intrarenal action of frusemide on renin secretion. However the experimental conditions in these studies were such that extrarenal factors cannot be completely ruled out.

Conclusive evidence for an intrarenal effect of frusemide on renin secretion is provided by the present study in which the isolated kidney of the rat was used. As seen with isoprenaline (Vandongen *et al.*, 1973) and diazoxide (Vandongen & Greenwood, 1975a), the response to a given dose of frusemide in this model varied considerably between experiments. One important determinant of this response appears to be the basal secretion rate, but other less apparent factors may also be involved. Although occlusion of the ureters alone will increase renin secretion (Kaloyanides, Bastron & Dibona, 1973) the basal renin values in this study are similar to values obtained with patent ureters recently reported from this laboratory (Vandongen & Greenwood, 1975b). It is apparent that the response to frusemide is not dependent on occlusion of the ureters as it is also observed when the ureter is patent. The similarity of the changes in renal perfusion pressure and flow rate in the frusemide and control experiments makes it unlikely that these are important in mediating the response in renin secretion. This is in contrast with other studies where the increase in renin secretion following intravenous frusemide was associated with a fall in renal blood flow and variable changes in mean arterial blood pressure (Meyer *et al.*, 1968; Vander & Carlson, 1969; Bailie *et al.*, 1973). It is conceivable however that frusemide produced localized changes in pressure and flow distribution (Birtch, Zakheim, Jones & Barger, 1967) in the isolated kidney which would not be detected by the methods used. The effect of frusemide on renin secretion was observed after ligation of the ureters and the presumed abolition of urine flow (Kaloyanides *et al.*, 1973) suggesting that the macula densa, which may influence renin secretion by detecting changes in tubular sodium movement (Vander, 1967), is not involved. Further evidence against an important role for this mechanism is the observation that renin secretion is stimulated by intravenous frusemide in dogs with a single non-filtering kidney (Corsini, Hook & Bailie, 1975) where the macula densa is presumed to be destroyed (Blaine, Davis & Witty, 1970).

It has been postulated that frusemide inhibits sodium transport at the macula densa site and that the resulting lowering of intracellular sodium concentration initiates renin release (Vander & Carlson, 1969). Whilst this proposal remains speculative, a direct effect of frusemide on the renin-producing cell should also be considered. Such an effect has been suggested for isoprenaline (Vandongen *et al.*, 1973), adrenaline and noradrenaline (Vandongen & Greenwood, 1975b), which in small doses activate only the renal $\beta$-adrenoceptors mediating renin secretion, and diazoxide a sodium-retaining thiazide derivative (Vandongen & Greenwood, 1975a). These agents have been shown to stimulate renin secretion in the isolated kidney independently of changes in renal perfusion pressure and flow rate. However, like frusemide (Hook, Blatt, Brody & Williamson, 1966; Ludens, Hook, Brody & Williamson, 1968; Ludens, Heitz, Brody & Williamson, 1970), these agents are potential renal vasodilators. It is conceivable therefore that the cellular mechanism involved in renin secretion is functionally related in some way to the contractor process and evidence has been previously given that calcium may occupy an important intermediary role in this relationship (Vandongen, Peart & Boyd, 1974; Vandongen & Peart, 1974).

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# CENTRAL MECHANISM OF VASOPRESSIN-INDUCED CHANGES IN ANTIDIURETIC HORMONE RELEASE

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1 Intracerebroventricularly (i.c.v.) administered vasopressin (0.001–1.0 u) in dogs anaesthetized with chloralose produced a dose-dependent increase in urine flow with a concomitant decrease in the levels of antidiuretic hormone (ADH) in jugular vein blood.

2 Higher doses of vasopressin (1.5–2.0 u, i.c.v.) on the other hand had an antidiuretic effect and produced an increase in blood ADH level.

3 Pretreatment (i.c.v.) with a $\beta$-adrenoceptor antagonist completely blocked the diuretic response of low doses of vasopressin (i.c.v.) but did not influence the antidiuretic response obtained with high doses.

4 Repeated administration of vasopressin (1.0 u, i.c.v.) induced tachyphylaxis; central catecholamine depletion with tetrabenazine significantly inhibited the vasopressin-induced diuretic response.

5 It is concluded that intracerebroventricular vasopressin-induced changes in ADH secretion are mediated through the release of catecholamines in the central nervous system.

## Introduction

In contrast to the well known peripheral antidiuretic effect of vasopressin (antidiuretic hormone, ADH), the administration of vasopressin into the lateral cerebral ventricles produced a diuretic response in normal dogs (Varma, Jaju & Bhargava, 1969) as well as in spinal transected cats (Nashold, Mannarino & Robinson, 1963). Since haemodynamic changes were observed concomitantly and ADH blood level changes were not measured by these workers, it is difficult to attribute the diuretic response following intracerebroventricular injection of vasopressin, to an inhibition of ADH release. The present study was undertaken to investigate the mechanism of the central vasopressin-induced changes in the release of antidiuretic hormone.

## Methods

The study was carried out in 38 mongrel dogs of either sex, weighing between 10–15 kg. The animals were fasted for 24 h but were allowed water *ad libitum*. They were anaesthetized with 10 ml/kg of warm 1% $\alpha$-chloralose in 0.9% w/v NaCl solution (saline) injected intravenously (Yoshida, Ibayashi, Murakawa & Nakao, 1965). To maintain hydration and anaesthesia, a continuous drip of saline containing 0.1% chloralose was delivered through a femoral vein at a rate just exceeding the rate of urine flow. In another set of experiments, in order to elicit the full antidiuretic effect of higher doses of intracerebroventricular (i.c.v.) vasopressin, the urine flow rate was kept at a higher level by the administration of dextrose-saline (Bhargava, Kulshrestha & Srivastava, 1972; Bhargava, Kulshrestha, Santhakumari & Srivastava, 1973).

Blood pressure was recorded on a kymograph from a carotid artery. Both the ureters were cannulated and connected to a photoelectric drop recording assembly to measure the urine flow. One of the jugular veins was cannulated with a polyethylene indwelling cannula directed towards the head end, to collect blood for ADH estimations. The ADH in the blood was extracted on a column of XE-64 resin by the method of Weinstein, Berne & Sachs (1960) as modified by Yoshida, Motohashi, Ibayashi & Okinaka (1963). This method of extraction was found to give a recovery of 80% ADH in controls and results were modified accordingly. The ADH in the eluate was estimated by the antidiuretic assay method in rats (Dicker, 1953). Arginine-vasopressin (Pitressin, Parke-Davis) was used as a standard in the bioassay.

Bilateral vagotomy, sino-aortic denervation, adrenalectomy and spinal cord transection at C2 level were performed by standard techniques in different experiments. Two or three hours after the surgical procedure, when the urine flow rate was constant over a period of 30 min, the drugs were administered through a cannula implanted in one of the lateral cerebral ventricles (Bhargava & Tangri, 1959). The

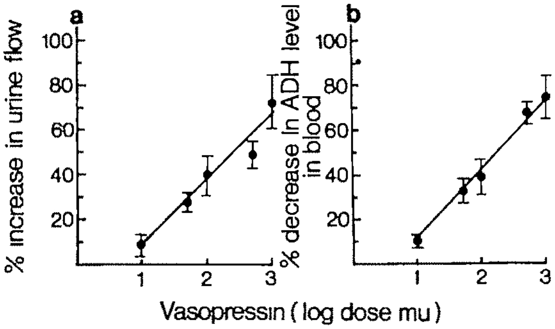

**Figure 1** Effect of graded doses (0.001–1.0 u) of vasopressin (i.c.v.) on (a) urine flow and (b) blood ADH concentration in dogs. Vasopressin induced a diuretic response accompanied by a corresponding fall in blood ADH titre

correct placement of the cannula was confirmed at autopsy. The total volume of fluid injected never exceeded 0.5 ml; in control experiments an equal volume of saline did not influence the urine flow.

### *Drugs*

The following drugs were used: vasopressin (Pitressin, Parke-Davis), *N*-isopropyl-*p*-nitrophenyl ethanolamine hydrochloride (INPEA, Selvi & Co.), (±)-propranolol hydrochloride (ICI), atropine sulphate (E. Merck), phenoxybenzamine hydrochloride (SKF) and tetrabenazine (Roche). The doses of compounds used refer to their salts.

## Results

### *Effect of vasopressin (i.c.v.) on urine flow and blood ADH level*

Vasopressin injected (i.c.v.) in graded doses of 0.001–1.0 unit, consistently produced an increase in urine flow that was associated with a corresponding fall in blood ADH concentration (Table 1). The log dose of vasopressin was found to be linearly related to the diuretic response or the fall in blood ADH titre in this dose range (see Figure 1). The diuretic response observed with 1.0 unit of vasopressin started within 10–20 min after injection, the peak effect was observed within 40–60 min and gradual recovery occurred in 90–120 min (Figure 2). With smaller doses (<1.0 unit) of vasopressin (i.c.v.) the diuretic response was of shorter duration depending upon the dose.

Higher doses of vasopressin (1.5–2.0 u, i.c.v.), on the other hand, induced an antidiuretic response (also shown in Figure 2), with a concomitant increase in blood ADH titre. With all the doses of vasopressin (i.c.v.) there was a rise in blood pressure with 1.0 unit of vasopressin, ranging from 5 to 20 mmHg. In all instances, the pressor response never lasted more than 10–20 minutes.

### *Effect of repeated (i.c.v.) administration of vasopressin*

Repeated injections of 1.0 unit vasopressin (i.c.v.) led to gradual decline in the diuretic response and after the third or fourth dose the diuretic response to vasopressin was not obtainable. However, smaller doses (0.001–0.5 u) of vasopressin, on repeated

**Table 1** Effect of intracerebroventricular vasopressin on urine flow and antidiuretic hormone (ADH) levels in the jugular vein blood of dog

| *Vasopressin (units i.c.v.)* | *ADH level (u/ml ± s.e. mean) in jugular vein blood at peak effect* | | *% Increase in urine flow at peak effect* |
|---|---|---|---|
| | *Control* | *After vasopressin (i.c.v.)* | |
| 0 01 | 15.2 ± 1.3<br>(*n* = 12) | 11.7 ± 0.9 | 8 |
| 0.05 | | 10.2 ± 1.4 | 28 |
| 0.10 | | 7.4 ± 0.6 | 40 |
| 0.50 | | 5 4 ± 0 5 | 50 |
| 1.0 | | 5.0 ± 0.6 | 74 |
| 2.0<br>(Hydrated dogs) | 4.2 ± 0.6<br>(*n* = 5) | 10.2 ± 3 6 | −34.6 ± 2.8* |

* Decrease in urine flow.

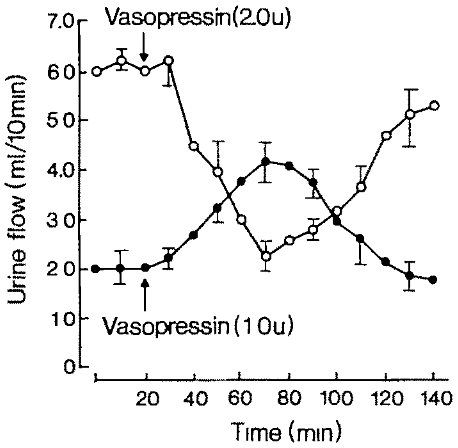

**Figure 2** Time-response curve of intracerebroventricular administration of vasopressin (1.0 and 2.0 u)-induced changes in urine flow. Vasopressin (i.c.v.) in a dose of 1.0 unit elicited a diuretic response which began within 10–20 min and reached a peak between 40–60 min; complete recovery occurred in 90–120 minutes. In contrast 2.0 units of vasopressin (i.c.v.), induced an antidiuretic response of a similar time course to that obtained with 1.0 unit.

administration did not produce significant tachyphylaxis.

### *Effect of surgical procedures on diuretic response to (i.c.v.) vasopressin*

Spinal cord transection (C2), bilateral adrenalectomy, bilateral vagotomy and/or sino-aortic denervation did not affect significantly the vasopressin (0.5 u and 2.0 u, i.c.v.)-induced diuretic and antidiuretic responses ($P > 0.05$) respectively. However, the pressor response obtained with vasopressin (i.c.v.) was completely blocked by bilateral adrenalectomy.

### *Effect of central drug pretreatment on (i.c.v.) vasopressin-induced diuretic response*

Prior central atropinization (2.0 mg i.c.v.) did not influence the diuretic response obtained with vasopressin (1.0 u, i.c.v.). Phenoxybenzamine (2.0 mg, i.c.v.), an $\alpha$-adrenoceptor blocking agent failed to prevent the vasopressin (1.0 u, i.c.v.)-induced increase in urine outflow or the fall in blood ADH concentration. However, the $\beta$-adrenoceptor blocking agents, INPEA (10 mg, i.c.v.) or propranolol (2 mg, i.c.v.) given 15 min beforehand, completely blocked the diuretic response to 1.0 unit of vasopressin (i.c.v.). Tetrabenazine (30 mg/kg i.p., given 4 h beforehand), a selective central catecholamine depletor, also blocked the vasopressin-induced response and fall in blood ADH titre ($P < 0.05$).

The antidiuretic response obtainable with higher doses of vasopressin (1.5 and 2.0 u) was not blocked by propranolol. Central $\alpha$-adrenoceptor blockade by phenoxybenzamine prevented the antidiuretic response to high (i.c.v.) doses (1.5–2.0 u) of vasopressin in 3 out of 5 animals; in the remaining 2 dogs this dose of vasopressin elicited a diuretic response after phenoxybenzamine pretreatment.

## Discussion

In this study, intracerebroventricular vasopressin (0.001 to 1.0 u) produced a dose-dependent increase in urine flow in dogs which was associated with a corresponding fall in ADH titre of jugular vein blood (Figure 1, Table 1). Only with higher doses of vasopressin (0.5–1.0 u, i.c.v.) was there also a rise in blood pressure (5–20 mmHg) which returned to a normal level within 20 minutes. The diuretic response of centrally administered vasopressin (1.0 u) started within 10–20 min, the peak effect was observed within 40–60 min and gradual recovery occurred in 90–120 minutes. From the time course of the diuretic response, it appears that haemodynamic factors (BP rise) are not responsible for increase in urine flow. Furthermore, the pressor response to vasopressin (i.c.v.) was not elicited in spinal transected or bilaterally vagotomized animals, whereas, prior spinal transection, bilateral vagotomy, sino-aortic denervation or adrenalectomy did not influence the diuretic response. These observations confirm that the vasopressin-induced diuretic response is of central origin and is mediated through central inhibition of ADH release.

Central receptors concerned in the control of antidiuretic hormone release were characterized by Bhargava *et al.* (1972). By studying the effects of intracerebroventricular administration of various cholinoceptor and adrenoceptor agonists and antagonists, it was concluded that central muscarinic cholinoceptors and $\alpha$-adrenoceptors are facilitatory and $\beta$-adrenoceptors are inhibitory for the release of ADH. In the present study, central atropinization or $\alpha$-adrenoceptor blockade with phenoxybenzamine (2.0 mg i.c.v.) did not block the vasopressin (i.c.v.)-induced diuretic response. Central pretreatment with INPEA (10 mg) or propranolol (2 mg), on the other hand, completely blocked the vasopressin (i.c.v.)-induced increase in urine flow or decrease in ADH titre of jugular vein blood ($P < 0.05$). These observations suggest that the (i.c.v.) vasopressin-induced diuretic response is mediated through central $\beta$-adrenoceptors.

In our experiments, repeated intracerebroventricular administration of vasopressin (1.0 u) induced tachyphylaxis. This would suggest an indirect central mechanism responsible for the inhibition of ADH release. Failure of vasopressin (i.c.v.) to induce a diuretic response in dogs pretreated with tetrabenazine (30 mg/kg i.p.), a selective central catecholamine depletor, supports an indirect mechanism of action. It is our contention that vasopressin (i.c.v.) releases catecholamines centrally and this is responsible for the activation of a central adrenergic mechanism for the inhibition of ADH release. In this connection, Statt & Chenoweth (1966) have demonstrated more than a 100% increase in catecholamine content of cavernous sinus blood following intravenous injection of vasopressin in dogs.

The antidiuretic response induced by high doses of vasopressin (1.5–2.0 u, i.c.v.) may arise either from a release of ADH or may be the result of peripheral leakage of the centrally administered vasopressin. The antidiuretic response to high doses of vasopressin (1.5–2.0 u, i.c.v.) did not bear any temporal relationship to the pressor response; the pressor response was inconsistent and of shorter duration (<20 min) as compared to the antidiuretic response. The antidiuretic response was associated with a concomitant rise in the ADH concentration in jugular vein blood and it was successfully antagonized by phenoxybenzamine (2.0 mg, i.c.v.). Furthermore, a local vasoconstrictor action of vasopressin cannot be held responsible for the antidiuretic effect. In 2 out of 5 dogs pretreated with phenoxybenzamine, the vasopressin-induced antidiuresis was converted to a diuretic response. It seems that central $\alpha$-adrenoceptor blockade is adequate to block the antidiuretic response and the subsequent diuretic response may be due to unmasking of a central $\beta$-adrenoceptor activation. These findings suggest a central action of vasopressin in eliciting the antidiuretic response as well. It appears that higher doses of vasopressin release larger amounts of catecholamines centrally and thereby predominantly activate the central $\alpha$-adrenoceptors which must be responsible for the antidiuretic response. This is consistent with the observation that low concentrations of catecholamines selectively activate $\beta$-receptors and higher concentrations are required to activate $\alpha$-receptors (Innes & Nickerson, 1970). In a previous study, intracerebroventricular injection of histamine was shown to induce an antidiuretic response which has been attributed to a massive release of catecholamines (Bhargava *et al.*, 1973). It appears that vasopressin also influences central ADH release through an adrenergic mechanism.

The foregoing results unequivocally demonstrate that the antidiuretic hormone (vasopressin) when introduced into the ventricular system of brain inhibits its own release and thereby elicits a diuretic response, i.e. an inhibitory feed back mechanism for ADH exists. ADH activity in the cerebrospinal fluid has been reported (Hoyle, 1933; Talanti & Kivalo, 1961; Heller & Ginsburg, 1966). However, it would be premature to suggest that this 'feed back' mechanism is operative in the abnormal physio-pathological states associated with high circulatory levels of ADH.

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# METABOLIC BASIS FOR THE SUPRA-ADDITIVE EFFECT OF THE ETHANOL-DIAZEPAM COMBINATION IN MICE

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1 The comination of ethanol and diazepam elicits additive or supra-additive pharmacological effects in animals and humans. Since the mouse appeared to be a reasonably good animal model for man, this species was examined to determine if a biochemical basis for these effects could be established.

2 The effect of ethanol (3 g/kg, orally), administered 0.5 h before [$^{14}$C]-diazepam (5 mg/kg, orally), on the pharmacokinetics of diazepam in male Swiss Webster mice was examined.

3 At 4, 6, 8 and 12 h after dosing, blood levels of $^{14}$C were higher in ethanol pretreated mice than in vehicle pretreated controls. A tissue distribution study indicated that ethanol had similar effects on levels of $^{14}$C in most tissues at 2, 6 and 12 hours.

4 Both pretreated and control mice eliminated approximately 25% of the dose of isotope in the urine and 50% in the faeces by 48 hours.

5 Biliary excretion was the major route of clearance in mice in which the bile ducts had been cannulated and ethanol reduced excretion at all time periods examined up to 12 hours.

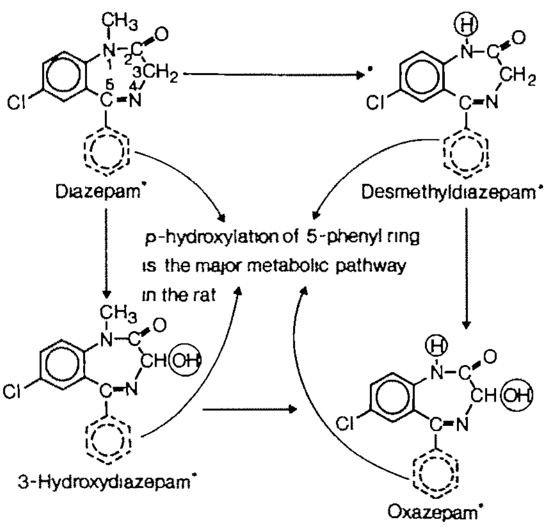
6 Oxazepam (the glucuronide in bile) and desmethyl diazepam were the major metabolites identified in bile, plasma and brain. Ethanol reduced oxazepam levels but increased desmethyl diazepam levels, suggesting that 3-hydroxylation of desmethyl diazepam was inhibited by ethanol.

7 The accumulation of the pharmacologically active desmethyl diazepam in the brains of ethanol pretreated mice offers an explanation for the supra-additive effect of the ethanol-diazepam combination on motor coordination.

## Introduction

Benzodiazepines have been used in the medical and dental field, because of their amnesic, sedative and muscle relaxant properties, for the symptomatic relief of tension and anxiety states. Recent reports have shown that their use has superseded that of barbiturates, making them the most commonly prescribed class of drugs (Shader, Greenblatt, Salzman, Kochansky & Harmatz, 1975; Kesson, Gray & Lawson, 1976). The metabolism of diazepam, one of the first marketed benzodiazepines, has been extensively studied in animals and man (Garattini, 1969; Schreiber, 1970; Zingales, 1973). The major diazepam degradation pathways are $N_1$-demethylation, $C_3$-hydroxylation and *para*-hydroxylation of the 5-phenyl ring (Figure 1); all except the latter leading to pharmacologically active derivatives (Garattini, 1969; Randall & Kappell, 1973).

Initial interaction studies carried out by Dundee & Isaac (1970) suggested that diazepam has no effect upon the blood concentration or dose of ethanol required to induce sleep, whereas chlorodiazepoxide was found to be an antagonist to ethanol. Other studies have not confirmed these findings but indicated that the combination of alcohol and diazepam elicits additive or supra-additive effects (Vapàatalo & Karppanen, 1969; Linnoila & Mattila, 1973a; Linnoila & Hakkinen, 1974; Mehar, Parker & Tubas, 1974; Morland, Setekleiv, Haffner, Stromsaether, Danielsen & Holst-Wethe, 1974). The basis for these additive or supra-additive effects of ethanol and diazepam in man has not been established. However, in a recent study from this laboratory it was found that ethanol elevated plasma and brain levels of diazepam by approximately 6-fold in the rat (Whitehouse, Paul, Coldwell & Thomas, 1975). When extrapolating these data to man, one is immediately confronted with the fact that the major route of metabolism in the rat is hydroxylation of the 5-phenyl ring, a pathway non-existent or of minor significance in man. Mouse and man share common major metabolic pathways for the biotransformation of diazepam ($M_1$-demethylation and $C_3$-hydroxylation; Schreiber, 1970). The effect of ethanol on the fate of diazepam was examined in the mouse to determine if a supra-additive or potentiating


Diazepam*
Desmethyldiazepam*
p-hydroxylation of 5-phenyl ring is the major metabolic pathway in the rat
3-Hydroxydiazepam*
Oxazepam*

• Pharmacologically active

**Figure 1** Major routes of diazepam biotransformation.

effect of diazepam in the presence of ethanol could be correlated with changes in the distribution and/or metabolism of diazepam.

## Methods

### *Animal treatment*

Male Swiss Webster mice (24–32 g) were acclimatized to the laboratory environment for at least one week before each experiment and food and water were supplied *ad libitum*. Before each experiment animals were fasted overnight (16 h) but allowed free access to water. Before (0.5 h) receiving diazepam (5 mg/kg, orally) mice were either given water 20 ml/kg orally (controls) or an aqueous (3.26 M) solution of ethanol equivalent to 3 g/kg. In the radioisotopic experiments [$^{14}$C]-diazepam labelled in the 5 position was given orally at a dose of 5 mg, 20 μCi/kg.

Common bile ducts were cannulated with heat-stretched polyethylene tubing (PE-10) while animals were under halothane anaesthesia. Mice were allowed 1 h following surgery to recover and were then dosed as described above, except that the dose of radiolabel was increased (5 mg, 40 μCi/kg, orally). Temperatures were monitored by rectal thermistor probes and body temperature maintained with heating lamps.

### *Sample collection and preparation*

Blood (5–50 μl) collected between 15 min and 48 h, tissues (50–100 mg of liver, heart, kidney, muscle, omental fat and brain), fluids (blood 10 μl, plasma 25 μl, and bile 5 μl) and the gastrointestinal tract (g.i. tract) and contents (200 μl of an aqueous homogenate made to 15 ml with distilled water) collected at 2, 6, 12 and 24 h from 5 control and 5 pretreated mice following [$^{14}$C]-diazepam administration, were digested at room temperature overnight with Soluene-100 (Packard Instrument Canada Ltd., Montreal Canada). Bile samples (5 μl) collected between 30 min and 12 h from mice with cannulated bile ducts were similarly processed. With the exception of the g.i. tract and contents which required 2 ml of Soluene-100, digestion was carried out with 1 ml of solubilizer. Samples were bleached with hydrogen peroxide and slight heating. At the end of the decolourization treatment, excess hydrogen peroxide was removed by heating and the resulting solution neutralized (acetic acid, 0.4 ml) and treated with water (1.5 ml) and Aquasol (15 ml, New England Nuclear, Boston Mass. 02118). No loss of [$^{14}$C]-diazepam radioactivity was encountered during the bleaching procedure.

Urine (200 μl), collected for 48 h from 3 control and 3 pretreated groups of 8 animals each, was assayed for radioactivity in Aquasol (15 ml). Faeces from the same mice were collected and suspended in 100 ml of distilled water. Aliquots (1 ml) were air dried, digested overnight and then treated with ethanol (0.25 ml) to prevent frothing during the bleaching process.

Radioactivity in plasma and brain samples was extracted with octanol and ethyl acetate, respectively and the percentage extraction of $^{14}$C determined as described previously (Whitehouse *et al.*, 1975).

Unless otherwise indicated, all samples were assayed for radioactivity by liquid scintillation counting using 15 ml of a toluene-based scintillation fluid (Liquifluor, New England Nuclear, Boston, Mass.) and a Nuclear-Chicago Mark II or Isocap 300 liquid scintillation counter as described by Thomas, Coldwell, Solomonraj, Zeitz & Trenholm (1972).

### *Separation of diazepam and its metabolites*

The nature of the radioactivity in plasma and brain extracts was examined by ascending thin-layer chromatography (TLC) on silica gel (Analab HF 250 micron plates) employing the chloroform : *n*-heptane : ethanol (10 : 10 : 1) system described by Schwartz & Carbone (1970). Developed TLC plates were scraped with an automatic TLC zonal scraper (Snyder-Kimble, Analabs Inc.) into serial 3 mm sections and transferred to vials containing 10 ml Aquasol. Native bile and bile treated for 16 h with Ketodase (β-glucuronidase, Warner-Chilcott) were similarly examined by TLC. In addition, samples from individual mice were pooled to give composite samples of bile, plasma and brain which were Ketodase-treated as before and separated by two-dimensional TLC (system A, chloroform : *n*-heptane : ethanol, 10 : 10 : 1; system B, isopropanol : ammonia, 20 : 1).

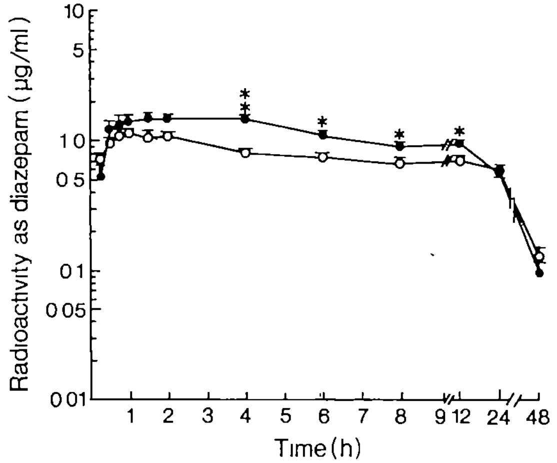


**Figure 2** Time course of blood radioactivity in mice following vehicle (○) or ethanol (3 g/kg, orally; ●) pretreatment, 0.5 h before $[^{14}C]$-diazepam (5 mg/kg, orally) administration. Values are means from five animals and are expressed as µg of diazepam per ml of blood. Vertical lines show s.e. means. $^{*}P<0.05$; $^{**}P<0.01$.

Oxazepam isolated from pooled bile samples was unequivocally identified by mass spectrometry. Pooled bile samples were extracted with diethyl ether, the extract was concentrated, spotted on silica gel plates and developed in system A with authentic standards. Silica gel in the area corresponding to oxazepam was scraped from the plate, extracted with diethyl ether and the extract rechromatographed with authentic standards using system B. Silica gel in the area corresponding to oxazepam was again re-extracted with diethyl ether. A mass spectrum was obtained by the direct probe insertion method at an electron voltage of 80 eV and a source temperature of 110°C on a low resolution mass spectrometer (Hitachi-Perkin-Elmer model RMS-4). Results were confirmed with a high resolution mass spectrometer (Varian MAT 311A) set on a fixed mass and operated at a source temperature of 250°C and electron voltage of 70 eV.

### *Motor coordination*

The rotacone, a modified rotarod, developed by Jacobsen (Danish Patent No. 117598, 1970) and described by Christensen (1973), was used to determine the effect of diazepam (5 mg/kg, orally), ethanol (3 g/kg, orally) and the diazepam-ethanol combination on animal motor coordination. Three groups of 8–9 mice were used in a cross-over design experiment and an interval of one week was allowed between each treatment. Motor coordination was examined at 0.5, 2, 4, 6 and 12 h after treatment with diazepam by the method of Christensen (1973). Results are expressed as activity ratios (AR):

$$AR = \frac{\text{Performance after drug administration}}{\text{Performance immediately before drug administration}}$$

### *Statistical analysis*

Data were tested for statistical significance by Student's $t$ test with $P<0.05$ being considered significant. In those cases in which percentages were compared, arc-sin transformed data were used (Snedecor & Cochran, 1967).

## Results

### *Effect of ethanol pretreatment on the distribution and excretion of $[^{14}C]$-diazepam-derived radioactivity*

Pretreatment with ethanol elevated blood concentrations of radioactivity at 4, 6, 8 and 12 h but did not statistically alter the area under the concentration–time curve (control, 25.20 ± 2.34 µg/ml × h; pretreated, 28.91 ± 1.69 µg/ml × h) or the $T_{\frac{1}{2}}$ (calculated from 12–48 h data) for elimination (control, 14.19 ± 1.48 h; pretreated, 10.96 ± 0.72 h; Figure 2).

Ethanol pretreatment increased the concentration of radioactivity from orally administered $[^{14}C]$-diazepam in several organs and tissues but reduced the radioactivity recovered in the bile (Table 1). Muscle, fat and heart tissues also contained more radioactivity in ethanol pretreated mice than in corresponding controls. Quantitation of the radioisotope in the g.i. tract (and contents) collected from vehicle pretreated mice indicated that 69.2 ± 4.1, 67.1 ± 2.6 and 47.7 ± 2.1% of the dose administered could be accounted for in the g.i. tract at 2, 6 and 12 h respectively. Corresponding samples collected from ethanol pretreated mice gave lower percentages (37.6 ± 2.5 ($P<0.001$), 52.4 ± 3.8 ($P<0.02$) and 59.9 ± 4.9). By 24 h all statistical differences between control and pretreated mice in the distribution of $^{14}C$ had disappeared.

Approximately 25% of the dose of radioactivity was eliminated from the body in the urine in 48 h and only at 12 h could a significant inhibitory effect of ethanol on the urinary excretion of $^{14}C$ be demonstrated (Figure 3). The major route of $^{14}C$ elimination was in the faeces (Figure 3), and this was attributed to biliary excretion. This was confirmed by examining the biliary excretion of radioactivity in mice with cannulated bile ducts (Figure 4). Approximately 65–75% of the dose administered to vehicle and ethanol pretreated mice was excreted in the bile in 12 h and at all time periods examined ethanol pretreatment statistically reduced biliary elimination of radioactivity (Figure 4).

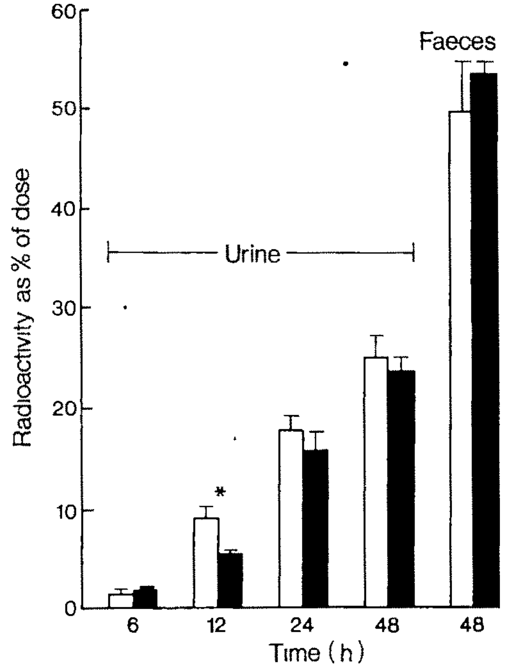


**Figure 3** Cumulative urinary and faecal excretion of radioactivity by mice pretreated with vehicle (open columns) or ethanol (3 g/kg, orally, solid columns) 0.5 h before[$^{14}$C]-diazepam (5 mg/kg, orally). Values are means from three groups of 8 mice each. Vertical lines show s.e means. $^{*}P<0.05$

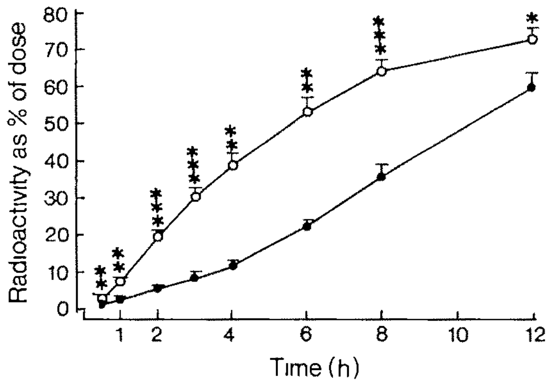


**Figure 4** Cumulative biliary excretion of radioactivity by mice pretreated with vehicle (O) or ethanol (3 g/kg, orally, ●) 0.5 h before [$^{14}$C]-diazepam (5 mg/kg, orally) administration. Values are means from 4 animals. Vertical lines show s.e means. $^{*}P<0.05$, $^{**}P<0.01$; $^{***}P<0\ 001$.

### *Metabolites of [$^{14}$C]-diazepam in bile, plasma and brain of vehicle and ethanol pretreated mice*

Three major areas of radioactivity were separated by TLC in system A of bile samples collected during the first 2 h after giving [$^{14}$C]-diazepam (Table 2). Ethanol pretreatment reduced significantly the percentage radioactivity that remained at or near the origin (area I). Chromatography of Ketodase-treated biles indicated that the major metabolite(s) of area II was excreted as a glucuronide and two-dimensional TLC (Figure 5) suggested it was oxazepam. Ethanol pretreatment also reduced significantly the excretion of this metabolite. Mass spectrometry of an extract of the tentatively identified oxazepam spot gave a fragmentation pattern similar to that of authentic oxazepam (m/e peaks at 286, 268, 259, 257, 239, 233, 229, 214, 205, 177, 151, 104, 77, and 51) and high resolution mass spectrometry gave a mass (286.0505 ± 0.0006) comparable to the theoretical mass of oxazepam (286.0509). An examination of samples of bile

**Table 1** Tissue distribution of radioactivity following [$^{14}$C]-diazepam (5 mg/kg, orally) administration to vehicle and ethanol (3 g/kg, orally) pretreated mice

| | | | *Time* | | | |
|---|---|---|---|---|---|---|
| *Tissue* | *Pretreatment* | n | *2 h* | *6 h* | *12 h* | *Units* |
| Plasma | Vehicle | 5 | 1.35 ± 0.15 | 0.72 ± 0.10 | 0.48 ± 0.05 | µg/ml |
| | Ethanol | 5 | 1 89 ± 0 11* | 1 29 ± 0 17* | 0.63 ± 0.04* | µg/ml |
| Brain | Vehicle | 5 | 2.10 ± 0.17 | 1.07 ± 0.12 | 0 77 ± 0 08 | µg/g |
| | Ethanol | 5 | 4.38 ± 0.30*** | 2.88 ± 0 37** | 1 26 ± 0.13** | µg/g |
| Kidney | Vehicle | 5 | 3 53 ± 0 31 | 2 25 ± 0 27 | 1.67 ± 0.19 | µg/g |
| | Ethanol | 6 | 6 87 ± 0 55*** | 5 08 ± 0 51** | 2.43 ± 0.30* | µg/g |
| Liver | Vehicle | 5 | 9.62 ± 0.55 | 6.28 ± 0.67 | 3.85 ± 0 44 | µg/g |
| | Ethanol | 5 | 12.53 ± 0 71** | 10.78 ± 1.08* | 5.47 ± 0.54* | µg/g |
| Bile | Vehicle | 3 | 0.15 ± 0.02 | 0.34 ± 0 04 | 0.31 ± 0.02 | mg/ml |
| | Ethanol | 4 | 0.04 ± 0.01** | 0.17 ± 0.03** | 0.23 ± 0.03 | mg/ml |

All values (means ± s e. mean) are expressed as µg or mg of diazepam per g or ml
$^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0\ 001$; when compared with corresponding results of vehicle pretreated mice.

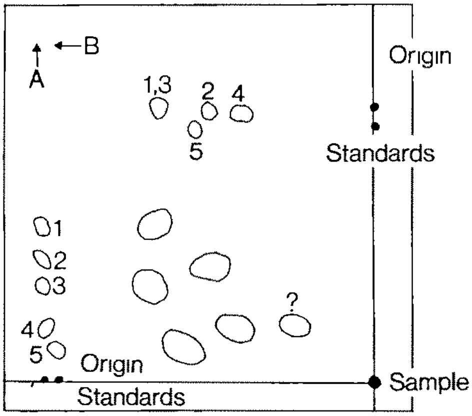


**Figure 5** Typical two-dimensional thin-layer chromatographic separation of radioactivity of bile obtained after the administration of [$^{14}$C]-diazepam on silica gel HF 250 micro plates. Authentic standards with their respective $R_F$s in system A (chloroform:*n*-heptane:ethanol, 10:10:1) and system B (isopropanol·ammonia, 20:1) were: 1 = diazepam, 0.35, 0.58; 2 = 3-hydroxy diazepam, 0.26, 0.40; 3 = desmethyldiazepam, 0.19, 0.58; 4 = oxazepam, 0.10, 0.31; 5 = 5-phenyl-*p*-hydroxy desmethyl diazepam, 0.05, 0.43.

obtained up to 4, 8 and 12 h indicated that the distribution of radioactivity between the different areas resembled that seen at 2 hours.

Two-dimensional TLC of 12 h bile samples with authentic standards, as shown in Figure 5, indicated that radioactivity could be further resolved. On the basis of migration in two solvent systems with authentic standards, area I contained a minor metabolite, 5-phenyl-*para*-hydroxydesmethyl diazepam, which accounted for 6.6 ± 0.7 and 5.2 ± 0.5% of the dose administered to vehicle and ethanol pretreated mice respectively. Area II contained oxazepam (42.2 ± 1.3 and 36.4 ± 2.4% of the dose in control and ethanol pretreated mice, respectively) and also an unknown component which represented less than 2.5% of the dose in both groups. Area III contained two metabolites which migrated similarly to desmethyldiazepam and 3-hydroxydiazepam; control and ethanol pretreated mice respectively excreted 3.6 ± 0.6% and 1.6 ± 0.4% ($P < 0.05$) of the administered dose as desmethyldiazepam and 0.8 ± 0.2% and 0.8 ± 0.3% as 3-hydroxydiazepam.

Thin layer chromatography of plasma and brain extracts using system A indicated that oxazepam and desmethyl diazepam were the major sources of radioactivity in the plasma (>65%) and brain (>85%). Using pooled extracts of plasma and brain and two-dimensional TLC with authentic standards this observation was confirmed. Ethanol pretreatment significantly increased the octanol extractability of radioactivity in plasma at 2 h (75.1 ± 4.0 to 94.7 ± 0.5%) and TLC of these extracts indicated that plasma levels of oxazepam were reduced whereas desmethyldiazepam levels were significantly elevated (Table 3). Chromatographic examination of ethyl acetate extracts of brain (>98% recovery) showed that brain concentrations of oxazepam and desmethyl diazepam mirrored the results obtained from corresponding plasma samples, with the exception, that concentrations were approximately 2-fold greater (Table 3).

### *Effect of ethanol pretreatment on motor coordination in mice following diazepam administration*

Oral administration of ethanol or diazepam alone did not significantly alter the performance of mice on the 'rotacone' from control values obtained immediately before dosing (Figure 6). However, the combination of ethanol and diazepam produced a supra-additive effect lasting for at least 6 hours. By 12 h the responses elicited from ethanol, diazepam and the

**Table 2** Thin-layer chromatographic separation of radioactivity from bile obtained for 2 h following [$^{14}$C]-diazepam (5 mg/kg, orally) administration to vehicle and ethanol (3 g/kg, orally) pretreated mice

| $R_F$ *range* | *Pretreatment* | *Native bile* | *Ketodase-treated bile* |
|---|---|---|---|
| Area I 0.00–0.07 | Vehicle | 19.17 ± 1.63 | 9.24 ± 1.07 |
| | Ethanol | 5.35 ± 0.91*** | 1.31 ± 0.21*** |
| Area II 0.07–0.11 | Vehicle | 0.29 ± 0.06 | 7.20 ± 0.78 |
| | Ethanol | 0.10 ± 0.01 | 1.77 ± 0.36** |
| Area III 0.12–0.22 | Vehicle | 0.14 ± 0.04 | 3.18 ± 0.45 |
| | Ethanol | 0.11 ± 0.05 | 2.53 ± 0.58 |

All samples were chromatographed on silica gel HF 250 micro plates using chloroform:*n*-heptane:ethanol (10:10:1). Values given are radioactivity recovered in the total bile over 2 h expressed as mean percentages of the dose ± s.e. mean from 4 mice.

** $P < 0.01$; *** $P < 0.001$ when compared with vehicle pretreated mice.

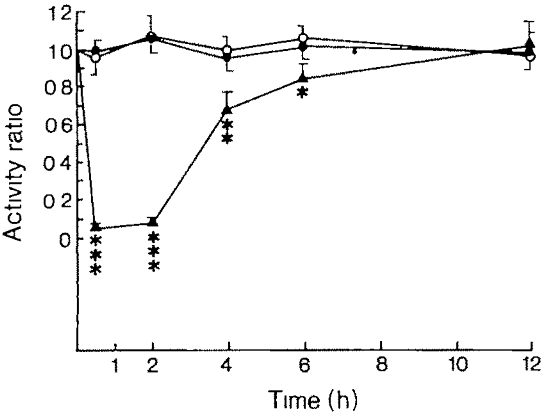


**Figure 6** Effect of diazepam (5 mg/kg, orally, ○), ethanol (3 g/kg, orally, ●) and the combination (▲) on animal coordination expressed as activity ratios (response of animal on the rotacone after treatment divided by response before treatment) Values are means from 26 mice used in crossover designed experiments. Vertical lines show s.e. mean $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

combination of ethanol and diazepam were comparable.

## Discussion

The supra-additive or potentiating effect of the ethanol-diazepam combination observed in mice (Figure 6) has been reported previously in man (Linnoila & Mattila, 1973a, b; Linnoila, Saario & Maki, 1974; Linnoila & Hakkinen, 1974; Morland *et al.*, 1974) and animals (Vapàatalo & Karppanen, 1969) but a pharmacological basis for these effects has not been established. Vuorinen, Heinonen & Rosenberg (1976) reported a study on the effect of ethanol on the duration of lidocaine narcosis in rats and mice. Lidocaine narcosis was prolonged by a single dose of ethanol of 1.5–3.0 g/kg. Since prolongation of the narcosis due to hypothermia had been precluded by the experimental design, the potentiating effects of ethanol were attributed to one or more of the following possibilities: (a) alteration in absorption or distribution, (b) synergism at the site of action and (c) inhibition of lidocaine metabolism.

Since the areas under the blood radioactivity–time curves (Figure 2) and the $T_{\frac{1}{2}}$ obtained from control and pretreated mice were not significantly different, potentiation by ethanol due to an increased absorption of diazepam could be excluded. The fact that excretion of total radioactivity in the urine and faeces (Figure 3) was not increased by ethanol pretreatment supported this contention.

Examination of bile collected from mice with cannulated bile ducts indicated that biliary excretion was the major route of [$^{14}$C]-diazepam clearance (Figure 4). The fact that by 12 h, 73.2 ± 3.4% of the dose of $^{14}$C could be accounted for in the bile of control mice with cannulated bile ducts (Figure 4) whereas only 50.2 ± 4.7% could be accounted for in the faeces at 48 h (Figure 3) suggests that diazepam metabolites undergo enterohepatic circulation in the mouse, a phenomena reported by Baird & Hailey (1972) for man. Ethanol pretreatment inhibited the 0–12 h cumulative biliary excretion of $^{14}$C (Figure 4) and the elevated blood (Figure 2) and tissue (Table 1) concentrations of $^{14}$C in ethanol pretreated mice are probably a consequence of this inhibition of biliary excretion.

Ethanol is known to inhibit the drug metabolizing enzyme system (Rubin & Lieber, 1968; Rubin, Gang, Misra & Lieber, 1970; Coldwell, Paul & Thomas, 1973; Mezey, 1976), thus preventing the elimination of lipid soluble material from the body. This possibility was examined. The major metabolite in bile was oxazepam glucuronide (Table 2), and ethanol inhibited the biliary excretion of this metabolite at 2, 4 and 8 hours. In addition the excretion of the radioactive metabolites, which remained at or near the origin after Ketodase treatment of bile, was also decreased by ethanol pretreatment at all time periods examined,

**Table 3** Effect of ethanol (3 g/kg, orally) on plasma and brain concentrations of oxazepam and desmethyl diazepam in mice 2 h after administration of [$^{14}$C]-diazepam (5 mg/kg, orally)

| *Metabolite* | *Pretreatment* | *Plasma (μg/ml)* | *Brain (μg/g)* |
|---|---|---|---|
| Oxazepam | Vehicle | 0 54 ± 0 03 | 1.10 ± 0.03 |
| | Ethanol | 0 31 ± 0.03** | 0.70 ± 0.03*** |
| Desmethyldiazepam | Vehicle | 0.32 ± 0.05 | 0.56 ± 0.05 |
| | Ethanol | 1.46 ± 0.09*** | 3.12 ± 0.17*** |

Octanol extracts of plasma and ethylacetate extracts of brain were chromatographed on silica gel HF 250 micron plates using chloroform.*n*-heptane:ethanol (10·10:1). Values are means ± s.e. mean of five observations.

$^{**}P<0.01$; $^{***}P<0.001$, when compared to vehicle pretreated controls.

suggesting that in addition to inhibition of oxazepam formation, biotransformation of [$^{14}$C]-diazepam to other polar end products was being inhibited. The increased octanol extractability of $^{14}$C from plasmas of ethanol pretreated animals indicated a higher percentage of radioactive lipophilic material in the plasmas of ethanol pretreated mice than in vehicle pretreated controls giving credence to the inhibitory effects of ethanol on diazepam metabolism. The fact that ethanol pretreatment reduced at 2 h the plasma levels of oxazepam (Table 3), but increased desmethyl diazepam concentrations suggests that the inhibition of diazepam metabolism occurred at the metabolic step at which desmethyl diazepam is 3-hydroxylated to give oxazepam. This inhibition of drug metabolism by ethanol has been attributed by Ozols (1976) to a reduction in the transfer of reducing equivalents to the microsomal oxidase system.

Synergism by ethanol with the effect of diazepam on the central nervous system is one possible explanation for the supra-additive or potentiating effects of the ethanol-diazepam combination. However, the major compound found in brain was not diazepam itself, but oxazepam in control mice and desmethyl diazepam in ethanol pretreated mice (Table 3). In mice desmethyl diazepam possessed a lower $ED_{50}$ (4 mg/kg, orally) for the rotarod test than either diazepam (6 mg/kg, orally) or oxazepam (7 mg/kg, orally) (Randall & Kappell, 1973) and brain levels of desmethyl diazepam were higher in ethanol pretreated mice than in controls (Table 3). Therefore the most likely interpretation for the supra-additive effects of diazepam in mice following ethanol administration is that the inhibition of metabolism causes accumulation of a metabolite (desmethyl diazepam) which, in addition to a higher pharmacological activity, also possesses a higher lipid solubility than oxazepam (as indicated by TLC mobilities in system A) resulting in a greater partitioning into lipoid tissue such as the brain.

The mouse metabolizes diazepam in a similar way to man and therefore is a reasonably good animal model for studying drug-diazepam interactions. Extrapolation from mouse to man is not always possible, but data from this study certainly indicate that a diazepam-ethanol interaction is a distinct possibility in man. Even though the therapeutic index of diazepam is large and death as a direct consequence of the diazepam-ethanol combination is not likely to occur with normal therapeutic doses, death and injury as an indirect result of the supra-additive pharmacological effects of this combination can be expected.

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# SELECTIVITY OF BLOCKING AGENTS FOR PRE- AND POSTSYNAPTIC $\alpha$-ADRENOCEPTORS

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1 Low frequency (0.1 Hz) electrical stimulation of the rat isolated vas deferens produced regular contractions that were inhibited by low concentrations of clonidine.

2 The inhibition of the vas deferens produced by clonidine was presynaptic in origin and involved $\alpha$-adrenoceptors.

3 Presynaptic $\alpha$-adrenoceptor antagonist activity was assessed by studying the effects of increasing concentrations of the antagonists on cumulative clonidine dose-response curves on the stimulated vas deferens.

4 Postsynaptic $\alpha$-adrenoceptor antagonist activity was assessed by comparison of control cumulative noradrenaline dose-response curves with those in the presence of increasing concentrations of antagonists in the rat anococcygeus muscle.

5 The results indicate that yohimbine and phentolamine are more potent in blocking presynaptic than postsynaptic $\alpha$-adrenoceptors. Phenoxybenzamine and prazosin block postsynaptic $\alpha$-adrenoceptors preferentially.

6 The findings support the view that presynaptic and postsynaptic $\alpha$-adrenoceptors differ in their sensitivity to $\alpha$-adrenoceptor antagonists.

## Introduction

There is considerable evidence to support the concept that sympathetic nerve terminals possess $\alpha$-adrenoceptors. Activation of these presynaptic $\alpha$-adrenoceptors inhibits, and their blockade potentiates, the release of noradrenaline by nerve impulses (Langer, 1974). There is also evidence that presynaptic and postsynaptic $\alpha$-adrenoceptors differ in their sensitivity to both agonists (Starke, 1972; Starke, Montel, Gayk & Merker, 1974; Starke, Endo & Taube, 1975b), and antagonists (Dubocovich & Langer, 1974; Thoenen, Hurlimann & Haefely, 1964; Starke, Borowski & Endo, 1975a).

The effects of several $\alpha$-adrenoceptor antagonists have been studied to assess their relative potencies at presynaptic and postsynaptic $\alpha$-adrenoceptors. Presynaptic $\alpha$-adrenoceptor activity was assessed on the isolated vas deferens of the rat, stimulated at low frequency. Postsynaptic $\alpha$-adrenoceptor activity was assessed on the isolated anococcygeus muscle of the rat.

## Methods

### *Rat vas deferens*

Male albino rats in the weight range 80–110 g were killed by cervical fracture. The vas deferens was dissected free from the surrounding connective tissue and suspended in an organ bath of 2 ml capacity. The tissue was bathed in Krebs solution of the following composition (mmol/l): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $KH_2PO_4$ 1.2, $MgSO_4$ 0.6, $NaHCO_3$ 25.0 and dextrose 11.1 which was maintained at 27°C and gassed with 95% $O_2$ and 5% $CO_2$. The bath fluid was renewed continuously with a peristaltic pump which delivered 3 ml/minute. Longitudinal contractions of the tissue were recorded isometrically with a Statham Gold cell transducer (model UC3) linked to a Smiths Servoscribe recorder. The intramural nerves of the vas deferens were stimulated by rectangular pulses of 3 ms duration 10–30 V at a frequency of 0.1 hertz.

### *Rat anococcygeus muscle*

Male albino rats in the weight range 200–250 g were killed by cervical fracture. The anococcygeus muscle (Gillespie, 1971) was exposed, dissected free from the surrounding tissues and suspended in a 5 ml organ bath. The tissue was bathed in Krebs solution which was maintained at 37°C and gassed with 95% $O_2$ and 5% $CO_2$. A Statham Gold cell transducer was used to measure contractions.

### *Quantitative analysis of $\alpha$-adrenoceptor antagonist activity*

Corticosterone 40 μM, desmethylimipramine 10 nM and propranolol 0.1 μM were present in the Krebs solution throughout the experiments outlined below.

### *Presynaptic activity*

Contractions of the stimulated vas deferens were inhibited by clonidine. By exposing the tissue to increasing concentrations of clonidine it was possible to construct cumulative dose-response curves. Following the production of two successive and comparable cumulative dose-response curves to clonidine, the effects of increasing concentrations of antagonists were studied. Antagonists were allowed a contact time of 15 min before initial challenge with clonidine.

### *Postsynaptic activity*

Noradrenaline stimulates postsynaptic $\alpha$-adrenoceptors of the rat anococcygeus muscle to produce a contraction. Noradrenaline (dissolved in Krebs solution) was added to the bath in 0.05 ml volumes to obtain cumulative dose-response curves. Antagonists were added to the bath only after two successive and comparable dose-response curves to noradrenaline had been obtained. Antagonists (maximum volume 0.2 ml) were allowed a contact time of 10 min before initial challenge with noradrenaline.

In both presynaptic and postsynaptic studies the methods of Van Rossum (1963) were used to calculate $pA_2$, $pD'_2$ and $pD_2$ values.

### *Drugs*

The following drugs were used in this study: bretylium tosylate (Burroughs Wellcome); clonidine (Boehringer Ingelheim); corticosterone (Sigma), desmethylimipramine hydrochloride (Geigy); guanethidine sulphate (CIBA); noradrenaline bitartrate (Koch Light Laboratories Ltd.); papaverine sulphate (Macfarlane Smith); phenoxybenzamine hydrochloride (SKF); phentolamine mesylate (CIBA); prazosin hydrochloride (Pfizer); propranolol hydrochloride (ICI) and yohimbine hydrochloride (Sigma). With the exception of prazosin, which was made up at a concentration of 1 mg/ml in warm 0.9% w/v NaCl solution (saline) containing 30% ethanol and corticosterone which was dissolved in propylene glycol, all drugs were dissolved in saline.

## **Results**

### *The rat vas deferens as a model for presynaptic $\alpha$-adrenoceptor activity*

Stimulation of the intramural nerves of the rat vas deferens at low frequency (0.1 Hz), 10–30 V, and 3 ms produced regular contractions of the tissue. The preparation was very stable with no spontaneous changes in resting tension and the contractions remained constant for at least 4–5 hours. The electrically induced contractions of the vas deferens were inhibited by low concentrations of clonidine hydrochloride (Figure 1). There was little variation in the sensitivity of different preparations to clonidine and clonidine dose-response curves could be constructed repeatedly, again with little change in sensitivity, in individual experiments. The contractions of the vas deferens were also inhibited by guanethidine sulphate (3 µg/ml), bretylium tosylate (10 µg/ml) and papaverine sulphate (100 µg/ml).

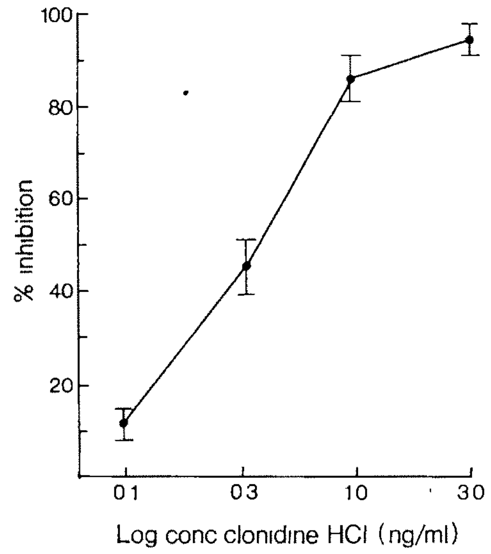

**Figure 1** The effect of clonidine on electrically induced contractions of the rat vas deferens. The results are the mean of 5 experiments. Vertical lines show s.e. means.

In order to determine whether clonidine was blocking sympathetic nerves or decreasing the sensitivity of the vas deferens itself, a second experiment was performed in which the tissue was stimulated directly. The stimulus parameters used were 100 V, 100 ms pulse width and a frequency of 0.1 hertz. Under these conditions clonidine, bretylium and guanethidine did not inhibit the contractions of the vas deferens whilst papaverine still caused inhibition of the 'twitch' response.

That clonidine, like bretylium and guanethidine, was interfering with nervous transmission and having little or no effect on the sensitivity of the vas deferens itself was confirmed in an experiment in which the vas deferens was contracted alternately by electrical stimulation (3 ms, 10–30 V, 0.1 Hz) and noradrenaline (1 µg injected into the Krebs stream). Clonidine hydrochloride (3 ng/ml) markedly antagonized the stimulation-induced contractions (Figure 2) but had little or no inhibitory effect on contractions evoked by noradrenaline.

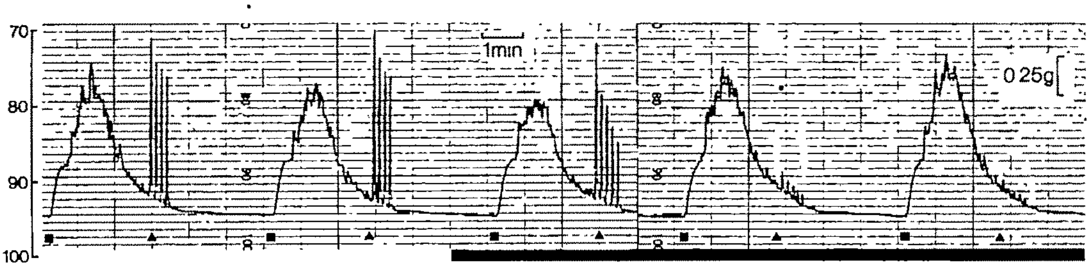

**Figure 2** The effect of clonidine on contractions of the rat vas deferens induced by noradrenaline and field stimulation: (■) noradrenaline 1 µg; (▲) electrical stimulation 0.1 Hz, 10–30 V, 3 ms, bar indicates presence of clonidine HCl 3 ng/ml.

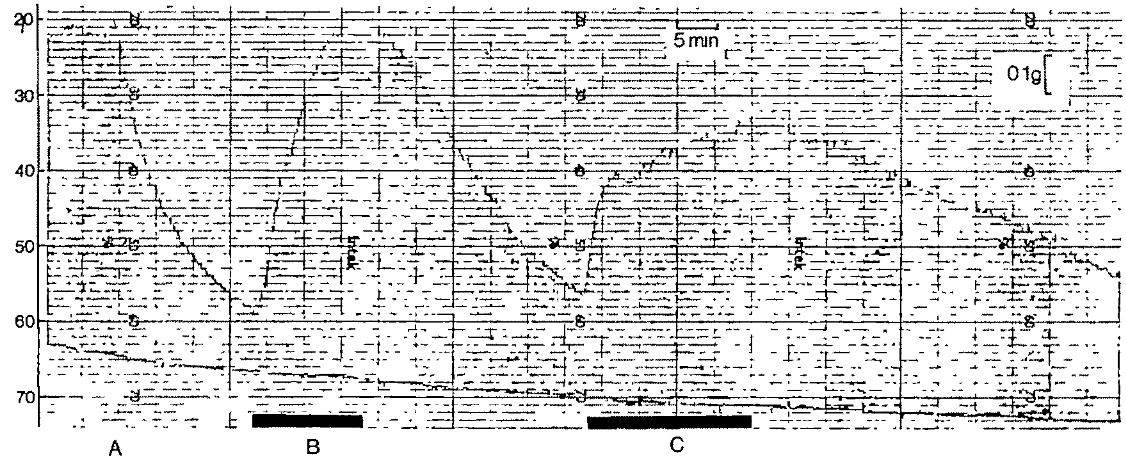

**Figure 3** The effect of antagonists on the inhibition produced by clonidine on the rat vas deferens. The vas deferens was stimulated at 10–30 V, 3 ms and 0.1 Hz; clonidine HCl was continuously present from point (A) at 3 ng/ml; (B) yohimbine HCl 100 ng/ml; (C) phentolamine mesylate 100 ng/ml.

Having established that clonidine affected nervous transmission, its mechanism of action was studied in the vas deferens stimulated at 10–30 V, 3 ms and 0.1 hertz. The inhibitory effects of clonidine could be antagonized competitively by phentolamine and yohimbine (Figure 3). The inhibition produced by bretylium, guanethidine or papaverine was not affected by phentolamine or yohimbine.

### *Determination of presynaptic α-adrenoceptor antagonist activity*

The rat vas deferens, stimulated at 0.1 Hz was used to construct cumulative dose-response curves to clonidine and the effects of the α-adrenoceptor antagonists yohimbine, phentolamine, phenoxybenzamine and prazosin upon them were examined.

**Table 1** Drug antagonism at the presynaptic α-adrenoceptor of the rat vas deferens

| *Agonist* | *Antagonist* | *Drug parameter* | n |
|---|---|---|---|
| Clonidine | | $pD_2 = 8.81 \pm 0.06$ | 11 |
| | Yohimbine | $pA_2 = 8.18 \pm 0.11$ | 6 |
| | Phentolamine | $pA_2 = 8.38 \pm 0.09$ | 6 |
| | Prazosin | $pA_2 < 6.62$ | 3 |
| | Phenoxybenzamine | $pA_2 < 7.54$ | 6 |

The Krebs solution contained corticosterone 40 µM, desmethylimipramine 10 nM and propranolol 0.1 µM. The results are expressed as the mean ± s.e. mean

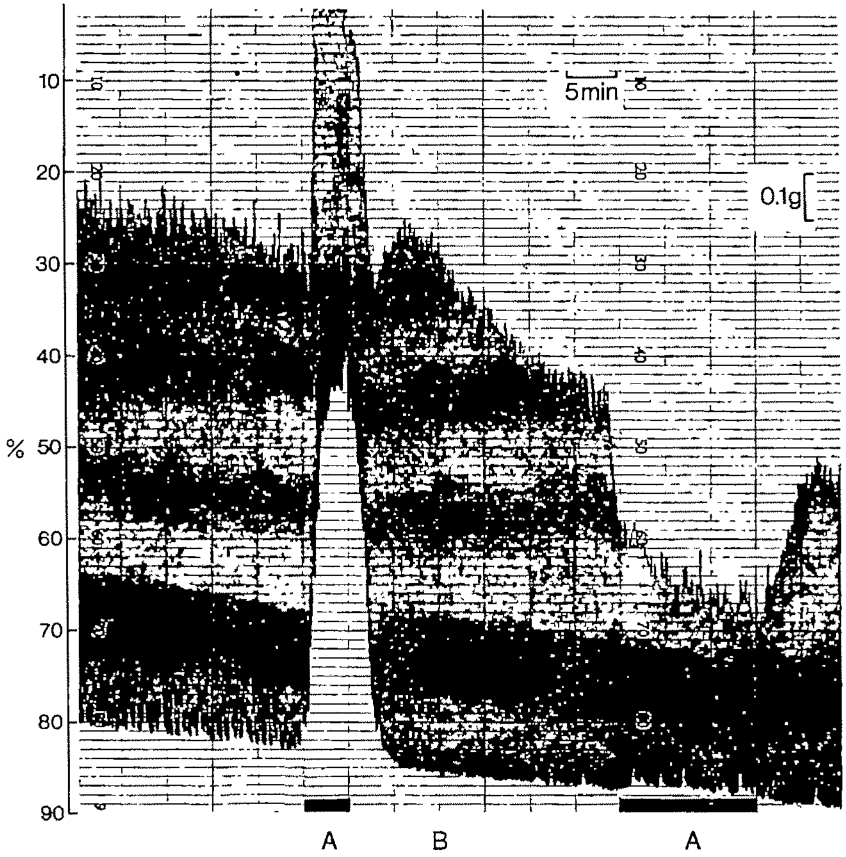

**Figure 4** The effect of noradrenaline on contractions of the rat vas deferens. The tissue was stimulated at 10–30 V, 3 ms, at 0.1 Hz; noradrenaline, 1 μg/ml at (A); prazosin HCl, 10 ng/ml, continuously present from (B).

At the concentrations used, yohimbine and phentolamine had no effect on the contractions of the vas deferens but both competitively antagonized the inhibition produced by clonidine. The $pA_2$ values for the compounds are shown in Table 1. There was no significant difference ($P > 0.05$) between the $pA_2$ values of phentolamine and yohimbine. Prazosin and phenoxybenzamine did not antagonize the inhibition produced by clonidine at the concentrations studied. Higher concentrations of prazosin and phenoxybenzamine (>100 ng/ml and >10 ng/ml respectively) reduced the contraction height of the vas deferens and consequently the interaction of clonidine with higher concentrations of prazosin or phenoxybenzamine could not be studied.

### *Determination of postsynaptic α-adrenoceptor antagonist activity*

The postsynaptic effects of the same antagonists in the rat anococcygeus muscle were studied using noradrenaline as the postsynaptic agonist. The log concentration-response curves for noradrenaline showed a parallel displacement to the right in the presence of phentolamine, yohimbine and prazosin. With phenoxybenzamine the maximum height attainable was reduced indicating that the antagonism was non-competitive at the concentrations studied. The $pA_2$ value (Table 2) of yohimbine was significantly different from that of phentolamine ($P < 0.001$) and prazosin ($P < 0.001$). The $pA_2$ value of phentolamine was significantly different from that of prazosin ($P < 0.05$).

From the experiments outlined in this and the previous section it was clear that prazosin had selectivity for postsynaptic α-adrenoceptors. This action was clearly demonstrated in a vas deferens preparation stimulated at 0.1 Hz, 20 V and 3 ms, in which an attempt was made to demonstrate the presynaptic agonist properties of noradrenaline. In this preparation noradrenaline produced no effect on the vas deferens at concentrations up to 100 ng/ml. At

1 μg/ml, noradrenaline produced a contraction of the vas deferens (Figure 4). When the effect of this concentration of noradrenaline was repeated in the presence of prazosin (10 ng/ml) the postsynaptic effect of noradrenaline was abolished and its presynaptic inhibitory effect unmasked.

## Discussion

The majority of investigations into the presynaptic control of transmitter release from sympathetic nerve endings have employed methods which involve the measurement of transmitter overflow using [$^3$H]-noradrenaline (Langer, 1974). The rat vas deferens subjected to low frequency field stimulation provides an alternative method for studying the effects of agonists and antagonists at presynaptic α-adrenoceptors.

Contractions of the vas deferens, induced by low frequency stimulation of the intramural nerves, could be inhibited either presynaptically by agents such as guanethidine or bretylium or by agents which relax smooth muscle such as papaverine. The contractions were also inhibited by clonidine; yohimbine and phentolamine had no effect on the twitch whereas high concentrations of both phenoxybenzamine and prazosin caused inhibition. Clonidine, like guanethidine and bretylium, produced its action presynaptically since in vas deferens preparations where the smooth muscle was stimulated directly all three agents were without effect whereas papaverine caused inhibition of the contractions. The presynaptic action of clonidine was confirmed in an experiment where clonidine, in concentrations that completely blocked contractions induced by stimulation of the intramural nerves of the vas deferens, had no effect on noradrenaline contractions in the same tissue.

Although the inhibitory effects of guanethidine, bretylium and clonidine were presynaptic in origin the mechanisms by which the inhibition of the twitch was produced were different. The inhibition of the 'twitch' produced by clonidine was competitively antagonized by α-adrenoceptor blocking agents e.g. yohimbine and phentolamine. The inhibition of the 'twitch' produced by either guanethidine or bretylium was unaffected by these blocking agents.

It was concluded from these studies that clonidine inhibited contractions of the rat vas deferens by a presynaptic action which involved α-adrenoceptors. When the tissue was used in conjunction with the rat anococcygeus muscle it was possible to compare activity at presynaptic and postsynaptic α-adrenoceptors. The low sensitivity of the vas deferens to noradrenaline coupled with considerable animal variation (Ambache & Aboo Zar, 1971) precluded its use for postsynaptic studies.

In the studies where relative potencies of α-adrenoceptor blocking agents were compared at presynaptic and postsynaptic α-adrenoceptors it was clear that the structural requirements for each receptor were different. The $pA_2$ value for yohimbine against clonidine on the vas deferens was significantly ($P < 0.001$) greater than its value against noradrenaline on the anococcygeus muscle. This result, although obtained by the use of a technique different from that used by Starke *et al.* (1975a), confirmed their finding that yohimbine was the first example of a preferential presynaptic α-adrenoceptor blocking agent. Phentolamine was also more active at presynaptic α-adrenoceptors than postsynaptic receptors, there being a significant difference ($P < 0.01$) between its $pA_2$ values against clonidine and noradrenaline.

Prazosin acted preferentially at the postsynaptic α-adrenoceptor. At the concentrations that it was possible to use, prazosin was devoid of presynaptic activity. There was at least a hundred-fold difference in the activity of prazosin at presynaptic and postsynaptic α-adrenoceptors.

Prazosin, a drug which is thought to have a peripheral action involving direct relaxation of vascular smooth muscle and sympathetic blockade (Pfizer, 1970), was included in this study because Constantine, McShane, Scriabine & Hess (1973) and Wood, Phelan & Simpson (1975) found that prazosin interferes with α-adrenoceptors. The results described here are consistent with these findings since prazosin

**Table 2** Drug antagonism at the postsynaptic α-adrenoceptor of the rat anococcygeus muscle

| *Agonist* | *Antagonist* | *Drug parameter* | n |
|---|---|---|---|
| Noradrenaline | | $pD_2 = 6.60 \pm 0.10$ | 12 |
| | Yohimbine | $pA_2 = 6.4 \pm 0\ 18$ | 6 |
| | Phentolamine | $pA_2 = 7.7 \pm 0\ 17$ | 6 |
| | Prazosin | $pA_2 = 8\ 2 \pm 0.11$ | 6 |
| | Phenoxybenzamine | $pD'_2\ 8\ 3 \pm 0.13$ | 9 |

The Krebs solution contained corticosterone 40 μM, desmethylimipramine 10 nM and propranolol 0.1 μM. The results are expressed as the mean ± s.e. mean.

competitively antagonized noradrenaline contractions on the rat anococcygeus muscle over a wide concentration range. In preparations where the intramural nerves of the vas deferens were stimulated at low frequency, prazosin, at relatively low concentrations ($\simeq$100 ng/ml), caused inhibition of the contractions. It was considered that this action was due to direct relaxation of smooth muscle; however, in vas deferens preparations where the smooth muscle was stimulated directly, prazosin at concentrations up to 10 µg/ml produced no inhibition of the contraction. It would appear therefore that the inhibitory effects of prazosin on contractions of the vas deferens were due to the fact that it is a preferential postsynaptic $\alpha$-adrenoceptor blocking agent.

Phenoxybenzamine was devoid of presynaptic activity at the concentrations that it was possible to use. Unlike the other antagonists studied, the interaction between noradrenaline and phenoxybenzamine at the postsynaptic $\alpha$-adrenoceptor was of a non-competitive nature.

In conclusion the results endorse the view that presynaptic and postsynaptic $\alpha$-adrenoceptors are different and, hence it is possible for $\alpha$-adrenoceptor blocking agents to have differential effects at these receptors.

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# PRENYLAMINE-INDUCED CONTRACTURE OF FROG SKELETAL MUSCLE

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1 Experiments were performed to determine the influence of prenylamine on excitation-contraction coupling in frog sartorius muscle.

2 Prenylamine (0.2–1.0 mM) produced a biphasic contracture in skeletal muscle characterized by an initial phasic and subsequent tonic contracture.

3 Neither dantrolene nor procaine blocked the prenylamine-induced contracture. Pretreatment with 100 mM $K^+$ blocked the phasic but not the tonic component of the prenylamine contracture.

4 Prenylamine produced a sustained increase in $^{45}Ca$ efflux at all concentrations that produce contracture. These concentrations of prenylamine also depressed the action potential, muscle twitch and resting potential.

5 Low concentrations of prenylamine (0.05 mM) which produced neither contracture, $^{45}Ca$ efflux nor $^{45}Ca$ influx, depressed the action potential, muscle twitch and $K^+$ contracture.

6 The results suggest that prenylamine not only alters calcium mobility but also membrane permeability to other ions.

## Introduction

Prenylamine (*N*-{3′-phenylpropyl-(2′)}-1,1-diphenylpropyl-(3) amine; Segontin) is one of a group of compounds which have been designated as organic calcium antagonists. At least two of these compounds, verapamil and prenylamine, have clinically useful cardiac antiarrhythmic properties which have been attributed to an action on membrane calcium conductance (Singh & Vaughan-Williams, 1972; Rosen, Ilvento, Gelband & Merker, 1974; Rosen, Wit & Hoffman, 1975). Several lines of evidence have indicated that these agents have rather specific effects on calcium mobility. First, the results of studies on cardiac atrial (Haas, Kern, Benninger & Einwächter, 1975) and ventricular muscle fibres (Kohlhardt, Bauer, Krause & Fleckenstein, 1972), cardiac Purkinje fibres (Cranefield, Aronson & Wit, 1974) and fibres in the sinoatrial and atrioventricular nodes (Wit & Cranefield, 1974) are consistent with the observation that these agents depress a slow inward calcium current. Secondly, both verapamil and its methoxy-derivative $D_{600}$ block a second, delayed component of calcium entry in squid axon (Baker, Meves & Ridgway, 1973). Thirdly, these agents have been shown to block excitation-contraction coupling in cardiac muscle (Fleckenstein, Tritthart, Fleckenstein, Herbst & Grün, 1969), smooth muscle (Haeusler, 1972) and skeletal muscle (Bondi, Kirsten & Hofmann, 1974). The apparent specificity with which these agents interfere with an inward flux of calcium ions, prompted us to investigate the effects of prenylamine on skeletal muscle.

Over the past few years, a considerable body of evidence has accumulated showing that activation of skeletal muscle proteins depends upon the release of calcium from the sarcoplasmic reticulum (Winegrad, 1970; Weber & Murray, 1973). However, the precise mechanism whereby depolarization of the transverse tubular (T-tubules) system is coupled to intracellular release of calcium is unknown. Two proposals for the coupling of sarcolemma excitation to the interaction of actin and myosin have been advanced. One suggestion (Bianchi & Bolton, 1967) involved the inward movement of a proposed 'trigger' calcium, located in the T-tubules and mobilized by T-tubular depolarization. This fraction of 'trigger' calcium regeneratively releases activator calcium from the sarcoplasmic reticulum and contraction ensues. An alternative possibility (Sandow, 1973; Podolsky, 1975; Sandow, Krishna, Pagala & Sphicas, 1975) involves an initial release of sarcoplasmic reticulum calcium caused by T-tubular depolarization and a subsequent regenerative release of activator calcium. The purpose of this investigation was to consider the effects of prenylamine on skeletal muscle with particular attention focused on the cellular calcium compartments with which prenylamine interacts. By studying several organic calcium antagonists, a variety of pharmacological actions on skeletal muscle may be revealed thereby providing additional clues to the coupling process described above.

## Methods

Sartorius muscles were dissected from pithed frogs (*Rana pipiens*) during all seasons of the year. After dissection, the muscles were equilibrated for 30–60 min at ambient temperature (22–24 °C) in oxygenated Ringer solution (normal Ringer solution) containing (mM): NaCl 110.8, KCl 2.0, $CaCl_2$ 1.8, $NaH_2PO_4$ 0.1, $NaHCO_3$ 2.02 and tris (hydroxymethyl)aminomethane 0.5 buffer adjusted to pH 7.2. Calcium-free Ringer was prepared without the addition of $CaCl_2$. Prenylamine lactate was dissolved in 100% ethanol to prepare a stock solution of 0.2 M and diluted 1 : 200 to 1 : 4000 in Ringer solution to prepare the final concentrations. The final ethanol concentrations never exceeded 0.5% (v/v). Control studies indicated that this concentration had no effect on the electrically-evoked muscle twitch. Solutions containing high concentrations of $K^+$ were prepared by an equimolar substitution of $K^+$-isethionic acid for the NaCl in the Ringer solution or Ringer solution containing prenylamine. In studies involving a second agent (i.e., dantrolene, procaine or caffeine), the test agent was added directly to the Ringer solution before use, and if necessary the pH was readjusted to 7.2 with HCl or NaOH.

### *Electrical and mechanical response studies*

The excised sartorius muscle was mounted horizontally in an acrylic plastic bath under a tension of approximately 2 grams. During the 30–60 min equilibration period, the muscle was subjected to direct twitch and tetanic stimulation. After the equilibration period, the Ringer solution was changed for one containing prenylamine. In studies involving pretreatment with dantrolene or procaine, the muscle was allowed to equilibrate with this agent for 30 min before the addition of prenylamine. In all experiments involving a second agent, paired sartorius muscles were used.

Isometric mechanical responses due to electrical stimulation or to contracture-inducing agents were measured with a Universal transducing cell (UC-4: Statham Inc.) with an attached load cell (Statham Inc), amplified and displayed on a polygraph. The areas under the tension curves were measured with a compensating polar planimeter (Keuffel and Esser). Supramaximal twitches (stimulus duration, 2.0 ms; frequency, 1 pulse/20 s) or fused tetani (200 ms train; duration 1 ms; frequency 500 Hz) were produced by field stimulation as previously described (Ross & Brust, 1968). (+)-Tubocurarine (70 μM) was added to the Ringer solution to block responses to acetylcholine released by electrical stimulation of nerve terminals.

The method for recording resting potentials and action potentials has been described previously (Kirsten & Kuperman, 1970a; Lustig & Kirsten, 1974). Sartorius muscles were mounted dorsal side upward and at rest length in an acrylic plastic bath. Transmembrane potentials were recorded by micropipettes (10–20 MΩ) filled with 3 M KCl and with tip potentials less than 5 mV. The micropipettes were coupled by a 3 M KCl bridge to a high impedance amplifier with capacitance feedback compensation (Bioelectric Inst., NF-1) and monitored on a storage oscilloscope. Several control impalements were made in each muscle before the Ringer solution was changed for one containing prenylamine.

In some experiments, the extracellular action potential and isometric twitch were recorded simultaneously. In these experiments, electrical stimuli (30–60 V) were provided by 0.15 ms pulses at a frequency of 1 pulse/s through a bipolar electrode (David Kopf Inst. SNEX-100). Stimuli were kept submaximal so that only superficial fibres were excited, preventing gross muscle movement which would interfere with consistency in recording. Extracellular action potentials were recorded differentially through teflon-coated bipolar silver wire electrodes (0.010 inch dia.), amplified by a bandpass amplifier (Ortec Inc., 4660), displayed on an oscilloscope and photographed.

### *Radioisotope studies*

The methods used for determining the uptake and efflux of $^{45}Ca$ (New England Nuclear Corp.) from sartorii were similar to those reported previously (Kirsten & Kuperman, 1970a; Lustig & Kirsten, 1974). In studies measuring $^{45}Ca$ uptake, one muscle was exposed for 15 min to $^{45}Ca$-Ringer (4 μCi/ml) while its contralateral mate was exposed to $^{45}Ca$-Ringer to which prenylamine (0.05 mM) was added. After $^{45}Ca$ exposure and blotting and rinsing with Ringer solution, the muscles were transferred consecutively to seven test tubes, each containing 3 ml of Ringer solution. The solutions bathing the muscle were changed and collected at 2, 5, 10, 15, 30, 60 and 90 min to wash out the extracellular calcium. At the conclusion of the washout period, the muscles were dissolved in acid and the radioactivity of the residue counted in a liquid scintillation spectrometer. After correction for decay and quenching, the data were expressed in terms of calcium influx. The $^{45}Ca$ space (ml per g) of the muscles multiplied by the calcium concentration of the Ringer solution (μmol per ml) determines the calcium uptake. The influx was obtained by dividing the calcium uptake (μmol per g) by the estimated surface area of the fibres, reported as 300 $cm^2/g$ (Bianchi & Shanes, 1959). The data were corrected for loss of $^{45}Ca$ from the slow compartment (Shanes & Bianchi, 1959) during the 90 min washout by using a correction factor of 1.42 (Bianchi, 1961).

Further $^{45}Ca$ uptake measurements were performed during $K^+$ depolarization. $^{45}Ca$ uptake was studied from paired muscles exposed for 3 min to $^{45}Ca$-Ringer solution and then exposed for 2 min to $^{45}Ca$-Ringer substituted with 100 mM $K^+$-isethionic acid in the presence (experimental) or absence (control) of prenylamine (0.05 mM). Following exposure, the residual $^{45}Ca$ content was determined as described previously.

Prenylamine-induced changes in the extracellular space were determined by using [$^{14}C$]-sucrose (4.3 mCi/mmol; New England Nuclear). After equilibration in oxygenated Ringer solution, paired sartorius muscles were transferred for a period of 30 min to Ringer solution (control) or Ringer solution (experimental) to which prenylamine was added. Directly following this treatment, the muscles were exposed for 30 min to the same solutions containing tracer amounts of [$^{14}C$]-sucrose. After a final blotting, the muscles were weighed and dissolved overnight in HCl. Samples of the tissue solvate were counted for [$^{14}C$]-sucrose as described above. Sucrose space was calculated as the ratio of the counts per min per g muscle (wet wt.) to counts per min per ml of the [$^{14}C$]-sucrose Ringer solution.

The radiocalcium procedures employed for the efflux data have been described previously (Kirsten & Kuperman, 1970a; Lustig & Kirsten, 1974). Paired sartorius muscles with their tendons removed, were loaded with $^{45}Ca$ by soaking for 3 h at ambient temperature in $^{45}Ca$ Ringer (4 μCi/ml). After loading, the muscles were rinsed with 20 ml of Ringer solution and gently blotted. The muscles were then placed in a chamber containing 3 ml Ringer solution. This collection fluid was changed for 3 ml of fresh solution at 10 min intervals through the entire washout period. At each 10 min interval, 1 ml of the total 3 ml washout fluid was emptied into a polyethylene scintillation vial and 15 ml of a modified Bray's scintillation fluor added (Kirsten & Kuperman, 1970a). At certain times during the washout period (to be indicated for each experiment), a test agent was added to the collection fluid. The muscle chambers were shaken during the entire washout and at no time during the washout period were the muscles removed from the chamber.

At the completion of 180 min of washout, the muscles were removed from the chamber, lightly blotted, and placed in silica crucibles. The muscles were dissolved in HCl overnight and aliquots of the tissue solvate were prepared for counting. The samples obtained at each 10 min collection period and the tissue solvate were counted in a liquid scintillation spectrometer. After correction for decay and quenching, the data for all experiments were expressed as rate coefficient curves. The rate coefficient, expressed in units of percent per min, is defined as the percentage of the average radioactivity in the muscle during the time of collection that has emerged per min of the collection period (Shanes & Bianchi, 1959). In some experiments, a relative rate coefficient curve was plotted. For these curves, the individual rate coefficients determined between 120 and 150 min of washout were averaged and taken as equal to 100%.

Comparisons between paired muscles were made with the use of Student's *t* test for paired comparisons. Differences were considered statistically significant at $P < 0.05$.

### *Drugs*

Prenylamine lactate was kindly supplied by Hoechst Pharmaceutical Co. and dantrolene by Eaton Labs of the Norwich Pharmacal. Co. Caffeine base, disodium edetate ($Na_2EDTA$) and procaine hydrochloride were obtained from Sigma Chem. Co. (+)-Tubocurarine was purchased from Organon Inc and $K^+$-isethionic acid from Eastman Chemical (Rochester, N.Y.).

## Results

### *Prenylamine-induced contracture*

The response of frog sartorius muscle to high concentrations (0.2–1.0 mM) of prenylamine was characterized by a biphasic contracture as shown in Figure 1a. The peak tension produced during the initial phasic and subsequent tonic contracture (second phase) increased with prenylamine concentration. Relaxation rate was faster with higher concentrations and relaxation to resting levels occurred with the drug still present in the bath. Figure 1b shows a plot of the peak tension during the contracture vs. the log of prenylamine concentration. The threshold concentration for a measurable response was approximately 0.1 mM. The area under the contracture curves (Figure 1c) varied in the same manner as the peak tension. The biphasic response due to 0.5 mM prenylamine was similar in contracture area to that produced by 100 mM $K^+$ (Putney & Bianchi, 1974).

### *Factors modifying the prenylamine contracture*

In an attempt to dissect the two phases of prenylamine contracture, we compared control contractures in one muscle with several pretreatment regimens for its contralateral mate. Dantrolene, a peripherally acting muscle relaxant (Snyder, Davis, Bickerton & Halliday, 1967), has recently been shown to be an effective blocker of excitation-contraction coupling (Ellis & Bryant, 1972; Putney & Bianchi, 1974).

Pretreatment with dantrolene, in a concentration double (16.6 μM) that used by Putney & Bianchi

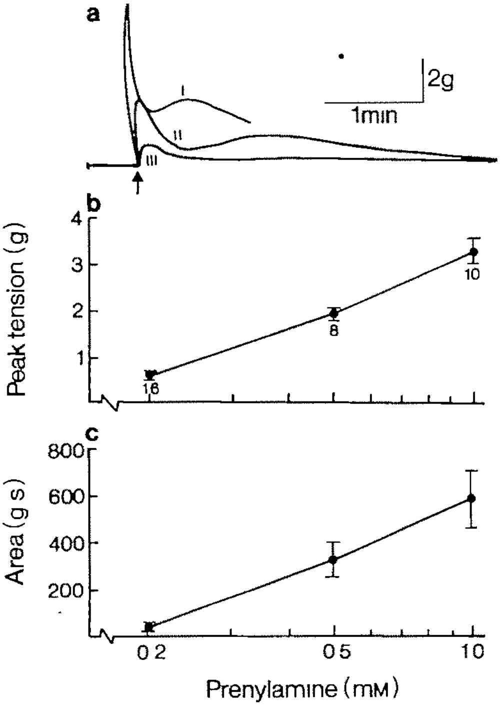

**Figure 1** Prenylamine contractures in skeletal muscle. (a) Rate of isometric tension development in frog sartorii exposed to prenylamine (arrow) in concentrations of: (i) 1.0 mM; (II) 0.5 mM; and (III) 0.2 mM. Prenylamine produces a biphasic response, which is characterized by an initial phasic (first phase) and a subsequent tonic contracture (second phase) The tracings were recorded on a curvilinear recorder and photographically superimposed. (b) Prenylamine log dose-response curve describing the contracture of sartorius muscles exposed to 0 2 mM, 0.5 mM and 1.0 mM prenylamine. The response is expressed as peak tension developed during the first phase of contracture. The number of muscles used for each concentration is indicated and the standard error is shown. (c) Plot of area under the prenylamine contracture for the muscles exposed to prenylamine at the concentrations shown. The number of muscles used with each concentration is the same as in (b).

(1974), had no effect on prenylamine contracture ($n=6$) compared with paired muscles treated with dantrolene alone.

Procaine, in a concentration (3.67 mM) which blocks both the contracture and associated $^{45}$Ca efflux induced by caffeine (Feinstein, 1963) and quinine (Suarez-Kurtz & Paumgartten, 1973), did not alter contractures produced by prenylamine.

Prenylamine-induced contractures were also studied in depolarized muscle. Potassium isethionate

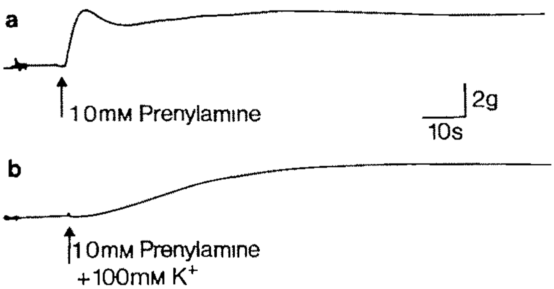

**Figure 2** Effects of high K$^+$ on prenylamine contractures (a) Isometric recording of a prenylamine contracture. (b) Contracture induced by prenylamine following 5 min pretreatment with 100 mM K$^+$ in the paired muscle. Resting tension returned to control levels before the addition of prenylamine in (b). High K$^+$ concentrations completely inhibit the first phase of the prenylamine contracture

(100 mM) was employed as the depolarizing agent in order to minimize changes in cell volume and intracellular ionic concentration. The effects of K$^+$ pretreatment on prenylamine contractures are illustrated in a typical experiment (Figure 2). Treatment with 100 mM K$^+$ completely abolished the first phase of the contracture but did not alter the second phase. The results of six experiments are summarized in Table 1. In contrast to the significant ($P<0.001$) depression of the first phase of the prenylamine contracture, neither the peak tension nor the latency to peak tension of the second phase was changed.

### *Effects on $^{45}$Ca movement*

Because of the firmly established role of calcium in excitation-contraction coupling (for review, see Fuchs, 1974) and previous reports of a calcium-antagonistic effect of prenylamine (Hasselbach, Balzer & Makinose, 1968; Van der Kloot, 1973), we determined the influence of prenylamine on $^{45}$Ca efflux and influx. Efflux of calcium from frog sartorius muscle can be considered as made up of two components (Shanes & Bianchi, 1959). The fast calcium component represents extracellular and loosely bound calcium; the slow component is considered to reflect calcium release from intracellular sites, presumably the sarcoplasmic reticulum. In six experiments, prenylamine (1.0 mM) added to the muscle chamber during the slow phase of $^{45}$Ca washout, produced an increase in the rate coefficient for $^{45}$Ca efflux as shown in Figure 3. Prenylamine caused a sustained increase in $^{45}$Ca efflux from whole muscle, an effect similar to that seen by the rigour-producing agents caffeine (Isaacson & Sandow, 1967), quinine (Isaacson, Yamaji &

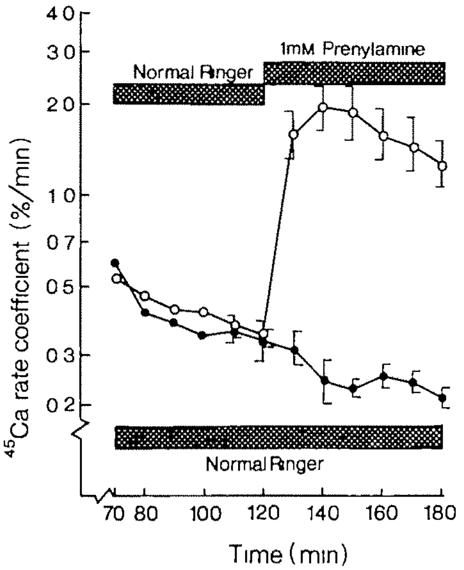

**Figure 3** Average change in rate coefficient of $^{45}$Ca efflux produced by 1.0 mM prenylamine (○) compared with paired untreated control muscles (●) in Ringer solution. The control muscles were washed out in Ringer solution for 180 minutes The experimental muscles were washed out in Ringer solution to which 1 0 mM prenylamine was added from 120 to 180 minutes. Notations at the top and bottom of the graph refer to the upper (○) and lower (●) curves, respectively. Each point is the mean of six ($n=6$) separate experiments. The standard error is given for the later portion of the curves

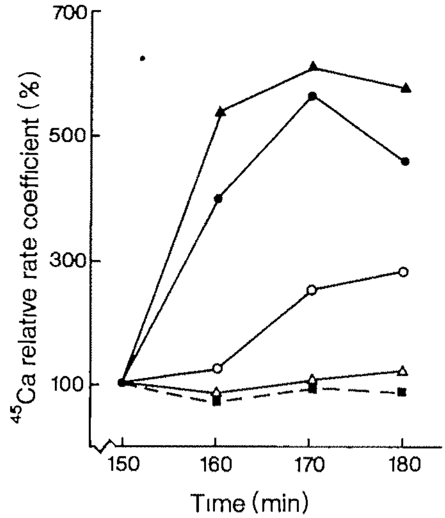

**Figure 4** The effect of prenylamine on $^{45}$Ca release from frog sartorius muscles. The data are plotted as a relative rate coefficient (%), where 100% is the average $^{45}$Ca efflux collected from 120 to 150 min of washout. Prenylamine was added at 150 minutes Each point is the mean of three experiments ($n=3$) and the concentrations of prenylamine used were (mM): 1.0 (▲); 0.5 (●), 0.2 (○) and 0 05 (△). No significant change ($P>0.2$) was observed for the muscles treated with 0.05 mM prenylamine and the control (■) muscles

Sandow, 1970) and *N*-ethylmaleimide (Kirsten & Kuperman, 1970b). However, rigour development was not observed with prenylamine.

The chelator EDTA is often employed to remove external or superficial calcium from the sarcolemma, thereby allowing the effect of an agent to be studied on the release of activator calcium from the sarcoplasmic reticulum. Previous studies (Bianchi, 1965) have demonstrated that EDTA does not penetrate into the muscle fibre and hence cannot chelate sarcoplasmic reticulum calcium. After 30 min pretreatment with EDTA, prenylamine still produced a doubling in the $^{45}$Ca released at the 120 min (i.e., preprenylamine) level. This increased $^{45}$Ca efflux following EDTA was similar to that observed with rigour producing agents.

Release of $^{45}$Ca was examined for four con-

**Table 1** Effects of 100 mM K$^+$ on contractures due to 1.0 mM prenylamine*

| | *Control*† | *Experimental*‡ | *C–E* | *Significance* |
|---|---|---|---|---|
| Peak tension First phase (g) | 3.13±0.36 | 0.00 | 3.13 | $P<0.001$ |
| Peak tension Second phase (g) | 3.38±0.35 | 2.59±0.24 | 0.79 | NS |
| Latency to peak Second phase (s) | 66.7±12.3 | 61.0±5.22 | 5.70 | NS |

* All values obtained for six pairs of sartorii and reported as mean ± s.e † Control contractures produced by 1.0 mM prenylamine. ‡ Experimental muscles were pretreated for 5 min with 100 mM K$^+$ before adding 1.0 mM prenylamine.

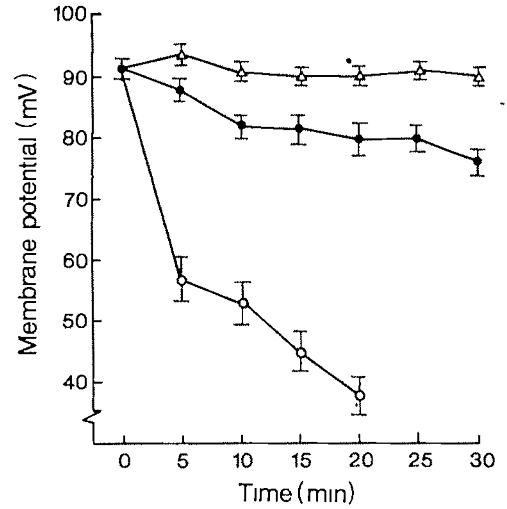

**Figure 5** Transmembrane potential of surface fibres before and after the addition of prenylamine at 0.05 (△); 0.2 (●) and 1.0 (○) mM. The average control resting potential is given at zero time. The values shown are pooled averages (±s.e.) of ±2.5 min from the time indicated. Each point is the average of 25 or more impalements ($n=25$) made on three muscles. At concentrations of 0.2 mM and 1.0 mM, prenylamine causes membrane depolarization At lower concentrations, prenylamine (0.05 mM) produces neither depolarization nor contracture.

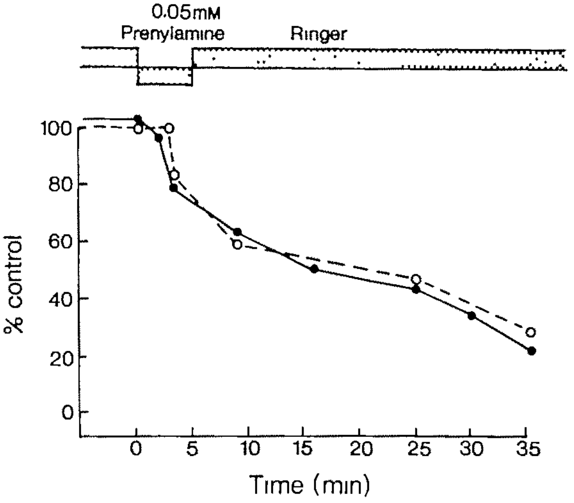

**Figure 6** Typical depression of muscle action potential (○) and twitch (●) by 0.05 mM prenylamine Inhibition of the extracellularly recorded action potential follows a time course similar to that observed for the electrically evoked muscle twitch At 5 min, the Ringer solution was restored, although recovery was not observed. Stimulation frequency: 1 pulse/20 seconds Higher concentrations of prenylamine produce more rapid depression of the muscle action potential and twitch.

centrations of prenylamine. We found that all concentrations of prenylamine which caused contracture also produced $^{45}$Ca release (Figure 4). Low concentrations of prenylamine (i.e., 0.05 mM) produced neither $^{45}$Ca release ($P>0.2$, as compared with controls) nor contracture.

Several investigators have suggested that the sustained calcium release produced by rigour-inducing concentrations of caffeine was attributable to progressive disruption of the transverse tubules and myofilaments (Huddart & Oates, 1970; Isaacson & Bárány, 1973; Lustig & Kirsten, 1974). In order to determine if the prolonged calcium release induced by prenylamine (0.2–1.0 mM) was secondary to a disruption of the sarcolemma and intracellular membrane system, we measured [$^{14}$C]-sucrose space in untreated and prenylamine-treated sartorius muscles. Using [$^{14}$C]-sucrose as a non-penetrating, non-electrolyte extracellular marker (Bozler, 1961), the average (±s.e.) sucrose space for sixteen muscles ($n=16$) was $0.26\pm0.01$ ml/gram. No significant change ($P>0.2$) in [$^{14}$C]-sucrose space was observed after 30 min treatment with 0.05 mM prenylamine ($0.27\pm0.01$ ml/g, $n=6$) although a significant ($P<0.001$) increase in [$^{14}$C]-sucrose space was seen after 1.0 mM prenylamine ($0.53\pm0.02$ ml/g, $n=10$). This marked increase in sucrose space may reflect a prenylamine-induced disruption of the muscle membrane at contracture producing concentrations.

In subsequent studies, we examined the effect of prenylamine (0.05 mM) on the $^{45}$Ca influx in resting muscles. Experimental muscles, pretreated with prenylamine before measurement of the $^{45}$Ca influx ($0.075\pm0.008$ pmol Ca cm$^{-2}$ s$^{-1}$), exhibited no significant difference from the control $^{45}$Ca influx ($0.074\pm0.013$ pmol Ca cm$^{-2}$ s$^{-1}$) measured in Ringer solution.

### *Membrane electrical properties*

As the first phase of the prenylamine contracture could be blocked by pretreatment with K$^{+}$, studies were performed to determine the transmembrane potential at three concentrations of prenylamine. At 0.2 or 1.0 mM, prenylamine caused a marked decrease in the resting potential of frog sartorius muscle (Figure 5). Resting membrane potential was not restored after extensive washing of the preparation. No significant change in resting potential was observed after non-contracture producing concentrations (0.05 mM) of prenylamine.

Although 0.05 mM prenylamine did not depolarize sartorius muscle, it did produce a simultaneous depression of the electrically-evoked muscle twitch and extracellular action potential as illustrated in Figure 6. Recovery was not evident when the preparation was returned to normal Ringer solution.

### *Potassium and caffeine contractures*

High K$^+$ and caffeine elicit muscle contracture by different mechanisms: high K$^+$ by causing membrane depolarization possibly through a 'trigger' calcium mechanism (Bianchi & Bolton, 1967) and caffeine by releasing bound calcium from the sarcotubular system (Isaacson & Sandow, 1967). With this in mind, studies were performed to determine the response of sartorius muscle to high K$^+$ or caffeine following treatment with 0.05 mM prenylamine. As shown in Table 2, prenylamine (0.05 mM) significantly reduced K$^+$ contracture tension (and ratio of peak tension to tetanus tension) and the $^{45}$Ca uptake produced by 100 mM K$^+$. In contrast, this low concentration of prenylamine had no significant effect on the rigour tension produced by 10 mM caffeine. Hence, prenylamine in the appropriate concentration had some selectivity in antagonizing K$^+$ but not caffeine-induced contractures.

## Discussion

The effects of prenylamine in a variety of systems can be explained as a direct or indirect consequence of an action on membrane calcium conductance (Fleckenstein *et al.*, 1969; Singh & Vaughan-Williams, 1972; Haas *et al.*, 1975). The possibility of prenylamine altering calcium mobility in skeletal muscle is, therefore, of considerable interest. At high concentrations (0.2–1.0 mM) prenylamine causes two phases of muscle contracture. The initial phasic contracture can be completely abolished by pretreating the muscle with K$^+$. Hence, the phasic aspect of the prenylamine contracture can be explained as a consequence of drug-induced depolarization of the sarcolemma and resultant increase in free calcium in the myoplasm.

A separate and distinct mechanism of action is responsible for the second, tonic contracture phase. The lack of effectiveness of either procaine (Inoue & Frank, 1962) or dantrolene (Putney & Bianchi, 1974) in antagonizing the prenylamine-induced tonic contracture would suggest that it is not mediated by an action on the sarcolemma or through activation of a 'trigger calcium' link (Bianchi & Bolton, 1967) in the excitation-contraction coupling process, respectively. An important clue to the effect of prenylamine in producing the second phase tonic contracture is that prenylamine causes a dose-related increase in $^{45}$Ca efflux from whole muscle. This $^{45}$Ca release is only observed at prenylamine dose levels which produce contracture and hence is similar to the $^{45}$Ca release reported for rigour producing agents (Isaacson & Sandow, 1967; Isaacson *et al.*, 1970; Kirsten & Kuperman, 1970b). As found by Hasselbach *et al.* (1968), prenylamine inhibits the active uptake of calcium in preparations of isolated sarcoplasmic reticulum vesicles. Presumably, the $^{45}$Ca release and the tonic contracture reflects a dose-dependent action

**Table 2** Effects of 0.05 mM prenylamine on $^{45}$Ca uptake and contractures induced by 100 mM K$^+$ or 10 mM caffeine*

| | *Control* | *Experimental†* | *C–E* | *Significance* |
|---|---|---|---|---|
| 100 mM K$^+$ | | | | |
| Peak tension (g) | 8.56 ± 0.58 | 4.28 ± 0.74 | 4.28 | $P<0.001$ |
| $\frac{\text{Peak tension}}{\text{Tetanus tension}}$ | 0.196 ± 0.01 | 0.092 ± 0.01 | 0.104 | $P<0.001$ |
| 100 mM K$^+$ | | | | |
| $^{45}$Ca uptake $\left(\frac{\text{nmol}}{\text{g}}\right)$ | 6.13 ± 0.54 | 4.10 ± 0.38 | 2.03 | $P<0.01$ |
| 10 mM Caffeine | | | | |
| Peak tension (g) | 23.9 ± 2.14 | 23.1 ± 2.13 | 0.8 | NS |
| $\frac{\text{Peak tension}}{\text{Tetanus tension}}$ | 0.48 ± 0.04 | 0.47 ± 0.05 | 0.01 | NS |

* Contracture studies obtained on eight pairs of sartorii. $^{45}$Ca uptake studies obtained on eleven pairs of muscles. All data are shown as mean ± s.e. † Experimental muscles were pretreated for 30 min with 0.05 mM prenylamine.

of prenylamine on the sarcoplasmic reticulum. The increase in [$^{14}$C]-sucrose space observed after exposure to 1.0 mM prenylamine may reflect a drug-induced disruption of subcellular structures at high concentrations as previously shown for caffeine (Huddart & Oates, 1970; Isaacson & Bárány, 1973).

In frog skeletal muscle, prenylamine induces a decrease in resting membrane potential, presumably due to an increased permeability to $Na^+$. Other studies with crayfish muscle (Van der Kloot, 1973) and frog skeletal muscle (Van der Kloot, Kita & Kita, 1975) indicated that prenylamine had no effects on transmembrane potential. The studies from Van der Kloot's laboratory on skeletal muscle were done at low concentrations (0.1 mM) and, hence, are similar to the results we obtained with 0.05 mM prenylamine. Unlike the decline in resting potential seen in this study with high concentrations of prenylamine, crustacean muscle is not depolarized. However, prenylamine (1.0 mM) does appear to block the calcium-dependent action potentials in this species (Van der Kloot, 1973).

In contrast to the rather diffuse actions observed at high concentrations, exposure of skeletal muscle to 0.05 mM prenylamine does not affect contracture, membrane potential, $^{45}$Ca efflux or $^{45}$Ca influx. The observed simultaneous depression of the action potential and twitch by prenylamine suggest a depressed excitability of the sarcolemma. This action of prenylamine is comparable to the local anaesthetic action (Lindner, 1960) and decrease in $K^+$ and $Na^+$ conductance (Van der Kloot, 1975) reported earlier. Verapamil, a papaverine derivative which reportedly also interferes with calcium mobilization and utilization (Fleckenstein, Grün, Tritthart & Byron, 1971) has also been shown to inhibit $Na^+$ currents in heart muscle (Shigenobu, Schneider & Sperelakis, 1974; Haas *et al.*, 1975) and have local anaesthetic properties (Singh & Vaughan-Williams, 1972; Bondi *et al.*, 1974; Van der Kloot & Kita, 1975). Hence, the calcium selectivity of these agents is lacking.

At 0.05 mM prenylamine exerts two kinds of effects on $K^+$-induced contractures: (1) it reduces contracture amplitude, and (2) it reduces the $^{45}$Ca uptake associated with the $K^+$ contracture. It is likely that these effects are exerted on a membrane site rather than a deeper structure as the caffeine-induced release of activator calcium from the sarcoplasmic reticulum (Isaacson & Sandow, 1967) was not altered by prenylamine. While it is well known that $K^+$ contractures are dependent upon extracellular calcium (Lüttgau, 1963) it would be difficult to argue for a selective action of prenylamine on membrane calcium. More probably, prenylamine, besides altering calcium fluxes, also interferes with $Na^+$ and $K^+$ fluxes at one or more sites in the excitation-contraction coupling process.

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# RESPONSES OF THE CARDIOVASCULAR SYSTEM OF THE RAT TO $\alpha$- AND $\beta$-ADRENOCEPTOR AGONISTS

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1 The effects of intravenously infused phenylephrine and isoprenaline upon the cardiovascular system of the rat anaesthetized with pentobarbitone, have been investigated.

2 Phenylephrine produces a dose-dependent rise in mean arterial blood pressure (MABP) that is due mainly to an increase in total peripheral vascular resistance (TPR), though at all doses tested cardiac output was invariably raised.

3 The increase in cardiac output was due in each instance to an increase in stroke volume, heart rate being unchanged. This increase in cardiac output is probably brought about by effects of phenylephrine on the capacitance vessels rather than by an effect on the heart.

4 Evidence is presented to show that the effects of phenylephrine are mediated largely by $\alpha$-adrenoceptors, but that $\beta$-adrenoceptors which affect TPR are also stimulated by the amine.

5 Isoprenaline produces a dose-dependent fall in MABP that is due entirely to a fall in TPR since the cardiac output increases.

6 Unlike phenylephrine, the increase in cardiac output obtained with isoprenaline was achieved by an increase in heart rate while stroke volume remained close to control values. It is contended that the augmented venous return required for the elevated cardiac output results in this case mainly from the isoprenaline-induced fall in TPR which enhances transfer of blood from arteries to the veins.

7 Evidence is presented to show that the effects of isoprenaline are mediated mainly by $\beta$-adrenoceptors.

8 Under the present experimental conditions the adrenoceptor-mediated cardiovascular changes are little modified reflexly by the arterial baroreceptors.

## Introduction

We have previously shown that the cardiovascular responses of the anaesthetized rat to noradrenaline infusion are unusual in so far as the resulting pressor response is mediated entirely by an increase in cardiac output with no alteration in total peripheral vascular resistance. That a catecholamine with predominantly $\alpha$-adrenoceptor stimulating properties should raise arterial blood pressure by this means was unexpected (Imms, Neame & Powis, 1974).

It is possible that the cardiovascular system of the rat contains adrenoceptors of types different from those of other species and which respond differently to stimulation by catecholamines. It is perhaps more likely that both $\alpha$- and $\beta$-adrenoceptors of the classical type are present throughout the cardiovascular system but that these have a functional balance of effects different from that which exists in other species.

To investigate these possibilities it was considered necessary to determine the cardiovascular effects of more selective $\alpha$- and $\beta$-adrenoceptor agonists in this species. The present paper describes the cardiovascular effects of the $\alpha$-adrenoceptor agonist phenylephrine and the $\beta$-adrenoceptor agonist isoprenaline.

## Methods

Male rats of an SPF Wistar derived strain and of body weight between 420 and 600 g were anaesthetized with pentobarbitone sodium (Sagatal, May & Baker, 70 mg/kg, i.p.). Supplementary doses of the anaesthetic (6 mg, i.p.) were given as required. The trachea was cannulated and all animals breathed room air spontaneously. Body temperature was maintained (±0.5 °C) by a thermostatically controlled heated table and an overhead lamp, and was monitored from a rectal thermometer.

### *Measurement of arterial blood pressure*

Arterial blood pressure was measured from a siliconed stainless steel cannula (i.d. 0.65 mm) containing heparinized saline (Heparin Injection B.P., Weddel Pharmaceuticals Ltd., 500 iu/ml 153 mM NaCl solution) introduced into the right femoral artery. The pressure was registered by a Bell & Howell type 4-327-L221 strain gauge transducer connected to the cannula by a short length (90 mm) of stiff nylon tubing. Mean arterial blood pressure (MABP) was extracted electronically by passing the amplified pulsatile blood pressure signal through a passive R-C network (Devices 3500) of time constant 18.5 seconds.

### *Measurement of heart rate*

Heart rate was recorded continuously from a rate meter (Devices 3531) triggered electronically by the amplified pulsatile waveform from the arterial blood pressure transducer.

### *Estimation of cardiac output*

Cardiac output was determined by a thermal dilution method (Fegler, 1954; Imms, Jones & Neame, 1971) in which 0.1 ml aliquots of 153 mM NaCl solution at room temperature were injected from a repeat injection apparatus (Hamilton PB600-10) into the right atrium through a catheter (o.d. 1.40 mm) advanced from the right external jugular vein. The difference in temperature between the injectate and the blood was obtained from a small thermistor of time constant approximately 100 ms (ITT U23US) mounted immediately behind the tip of the jugular venous catheter. The temperature of the aortic blood was recorded continuously from a second thermistor of similar characteristics mounted at the tip of a fine nylon tube (o.d. 0.93 mm) advanced into the aortic arch from the left common carotid artery. The thermal pulse resulting from the injection of 0.1 ml of saline at room temperature (20–24 °C) into the right atrium was monitored as it passed the aortic arch thermistor.

The signal was passed through a Devices 3553 conditioning unit to a Devices •3550 d.c. chopper amplifier. The curve described was integrated with a Devices 3630 integrator and the area encompassed by the dilution curve was extracted from the integral. The integral was evaluated at the point where the recorded dilution curve, corrected for non-exponential decay, returned to the baseline. This value was incorporated into the formula given by Hanwell & Linzell (1972) to obtain a value for cardiac output in ml/minute. A more detailed description of the method used is published elsewhere (Neame, Powis & Imms, 1977).

This technique for estimating the area beneath the thermal dilution curve was compared with the methods described by Hamilton (1962) and Williams, O'Donovan & Wood (1966). All three methods were used to derive cardiac output from each of six randomly selected curves recorded during different experimental situations. The three results calculated from each curve deviated from the mean value by no more than 5%.

### *Recording*

Arterial blood pressure, mean arterial blood pressure, heart rate, jugular and aortic temperatures and the integral of the aortic temperature change were displayed continuously on moving paper with a Devices M19 eight channel recorder.

### *Infusion of adrenoceptor stimulating drugs*

Solutions of phenylephrine hydrochloride (Phenylephrine Injection B.P., Boots Co. Ltd.) or isoprenaline sulphate (Macarthys Ltd.) were infused through a nylon catheter inserted into the left femoral vein with a roller pump (Type MHRE7; Watson-Marlow, Falmouth, Cornwall). The concentration of the solutions used was chosen to ensure that the required amounts of the drugs (700, 1400, 2000 and 5000 ng/min phenylephrine or 20, 50, 100 and 500 ng/min isoprenaline nominal) could be infused at a flow rate between 0.10–0.15 ml/minute. The mean amounts of phenylephrine administered at each dose level in the present series of experiments were 724 ± 12 ng/min (mean ± s.d.), 1382 ± 36 ng/min, 2202 ± 123 ng/min and 4773 ± 67 ng/min while those of isoprenaline were 21.5 ± 0.6 ng/min, 49 ± 2 ng/min, 122 ± 9 ng/min and 595 ± 43 ng/minute.

### *Experimental procedure*

The animals used constituted four experimental groups: phenylephrine was administered to one group (13 animals) at dose levels 700 and 1400 ng/min and to another group (16 animals) at 2000 and 5000 ng/minute. Into the animals of the third group (14 animals), isoprenaline was infused at 20 and 50 ng/min while into those of the fourth group (17 animals) isoprenaline was infused at 100 and 500 ng/minute.

A standard protocol was adopted for all experiments. On completion of surgery, heparin (500 iu/kg body weight) was given intravenously to all animals.

After each animal had stabilized for 5 min, six control cardiac output determinations were made after which the infusion of either phenylephrine or isoprenaline at the lower dose was started. After 5 min when arterial blood pressure and heart rate were invariably steady, six further determinations of cardiac output were made. The higher dose of the amine was then infused and a further series of six thermodilution curves were obtained when a steady state had been established. Infusion was then stopped and after 5–10 min, or when MABP and heart rate had returned to their resting levels, six control determinations of cardiac output were made.

Following this control period, in approximately half the animals of each group the $\beta$-adrenoceptor blocking agent, propranolol hydrochloride was administered (Inderal, ICI; 1 mg/kg, i.v.), while in the remaining animals of each group a similar volume of 153 mM NaCl solution (1 ml/kg, i.v.) was given. After a further 5 min stabilization period, the four part schema described above was repeated.

### Calculations

A mean value for cardiac output ±s.d. during each stage of the experimental procedure was calculated from each series of six thermodilution curves and expressed in ml/minute. The s.d. was usually about 3% of the mean value for cardiac output calculated from the six determinations; it was never greater than 7.5%. There was no significant relationship between resting cardiac output and body weight of the 60 animals used (cardiac output = 0.096 body wt + 47.1, $r=0.296$; $P \simeq 0.05$). Total peripheral vascular resistance was calculated as:

$$\frac{\text{MABP (mmHg)}}{\text{Cardiac output (ml/min)}} \times 10^3$$

and expressed in mmHg $\text{ml}^{-1}\,\text{min}^{-1}\,.\,10^3$ (PRU).

Stroke volume was calculated as the dividend of cardiac output and heart rate and expressed in ml/beat.

### Expression of results

Absolute values for the measured or derived cardiovascular variables under control conditions are given in Table 1. The responses obtained with either phenylephrine or isoprenaline infusion have been expressed in two ways:

(i) In order to compare visually the overall cardiovascular response to infused phenylephrine with that produced by isoprenaline, the mean changes in each variable produced by either drug were calculated as percentage changes from the relevant control value (Figure 1).

(ii) In the written results section and in Figures 2 and 3 the cardiovascular responses obtained in each animal with either phenylephrine or isoprenaline were calculated in terms of increments or decrements from control values, and the mean responses of the animals comprising each group were expressed as mean change ± s.e. mean.

The control values from which the changes in recorded or derived cardiovascular variables were calculated were obtained by interpolation between pre-infusion and post-infusion controls.

### Statistical analysis

A paired $t$ test was used to evaluate the significance of the cardiovascular responses obtained with either phenylephrine or isoprenaline. The value for $t$ is given by the dividend of the mean increment or decrement calculated according to (ii) above, and its s.e. mean. To compare the dose-response relationship of infused catecholamine after propranolol administration with that after saline (Figures 2 and 3) a chi-squared test was used.

**Table 1** Mean control values (±s.d.) for measured and derived cardiovascular variables in 60 animals divided into four experimental groups

| Group | n | *Cardiac output (ml/min)* | *Blood pressure (mmHg)* | *Heart rate (beats/min)* | *TPR (PRU)* | *Stroke volume (ml/beat)* |
|---|---|---|---|---|---|---|
| 1. $P^{700}_{1400}$ | 13 | 94.1 ± 12.1 | 125.5 ± 12.2 | 397 ± 23.1 | 1364 ± 281 | 0.238 ± 0.035 |
| 2 $P^{2000}_{5000}$ | 16 | 96.2 ± 23.2 | 129.6 ± 18.1 | 397 ± 26.3 | 1434 ± 434 | 0.245 ± 0.068 |
| 3. $I^{20}_{50}$ | 14 | 96.9 ± 13.2 | 133.3 ± 9.4 | 398 ± 24.8 | 1399 ± 212 | 0.243 ± 0.028 |
| 4. $I^{100}_{500}$ | 17 | 93.6 ± 18.0 | 130.7 ± 11.2 | 401 ± 34.9 | 1441 ± 398 | 0.234 ± 0.043 |
|  | 60 | 95.2 ± 17.1 | 129.9 ± 12.9 | 398 ± 27.4 | 1413 ± 343 | 0.240 ± 0.046 |

Under the column headed *Group*, P signifies phenylephrine infused and I signifies isoprenaline infused. The numbers following either letter denote the doses (ng/min) of each substance to be subsequently infused into the animals comprising the group

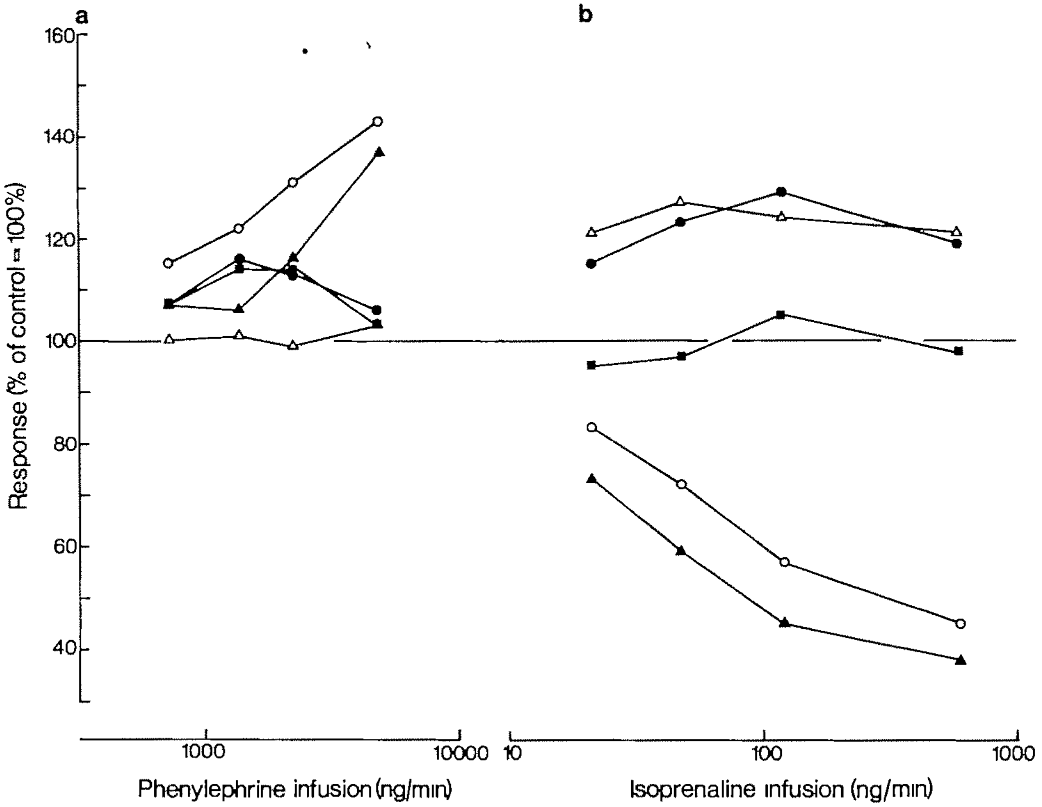

**Figure 1** The cardiovascular responses of the pentobarbitone anaesthetized rat to infused (a) phenylephrine and (b) isoprenaline The change in each measured or derived variable during infusion is expressed as a percentage of its control value. (○) Mean arterial blood pressure; (●) cardiac output; (△) heart rate; (▲) total peripheral resistance; (■) stroke volume.

## Results

### *Control data*

The mean body weight of the 60 animals used in the present series was 502 ± 53.2 g (mean ± s.d.). The mean body temperature of these animals measured after completion of surgery was 38.1 ± 0.93 °C.

These 60 animals were divided into four experimental groups: the group mean values for body weight and temperature did not differ significantly from the overall mean values given above. The mean resting values for both the measured and derived cardiovascular variables in each of the four experimental groups are given in Table 1.

### *Effects of phenylephrine infusion*

Significant pressor responses occurred at all doses infused (Figure 1a); as the rate of infusion was increased, MABP increased in a linear fashion. The increase in MABP in each case was due to increases both in TPR and in cardiac output, although at the two highest levels of infusion increases in TPR made a progressively larger contribution to the pressor response while the increase in cardiac output reached a maximum at an infusion rate of 1400 ng/min and then declined.

At the lowest rate of infusion MABP rose by 17.3 ± 2.2 mmHg (mean ± s.e. mean); an increase brought about by an increase in cardiac output of 6.9 ± 3.1 ml/min and an increase in TPR of 82 ± 36 PRU (Figure 2). At the higher rates of infusion (1400, 2000 and 5000 ng/min) MABP increased by 24.7 ± 1.5, 35.8 ± 3.9 and 48.6 ± 4.6 mmHg respectively. These rises were due to increases in TPR of 54 ± 38, 211 ± 50 and 486 ± 72 PRU and in cardiac output of 14.5 ± 3.0, 12.5 ± 2.6 and 5.5 ± 2.6 ml/minute. At no rate of infusion did the heart rate alter significantly (Figure 2).

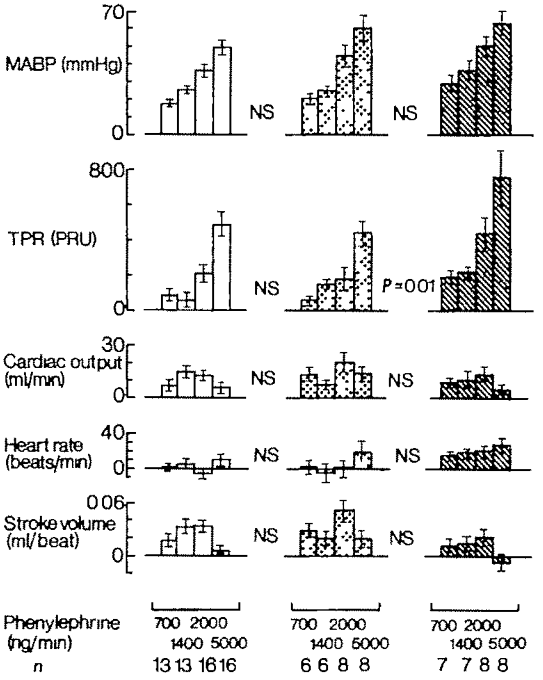

**Figure 2** Mean cardiovascular changes produced by phenylephrine infused at four rates before (open columns) and after administration of either saline (dotted columns) or propranolol (hatched columns). Vertical lines show s.e. mean. The responses were compared with a chi-squared test and differences together with levels of significance are given between the groups of responses. NS signifies no difference between the groups at the 5% level of significance

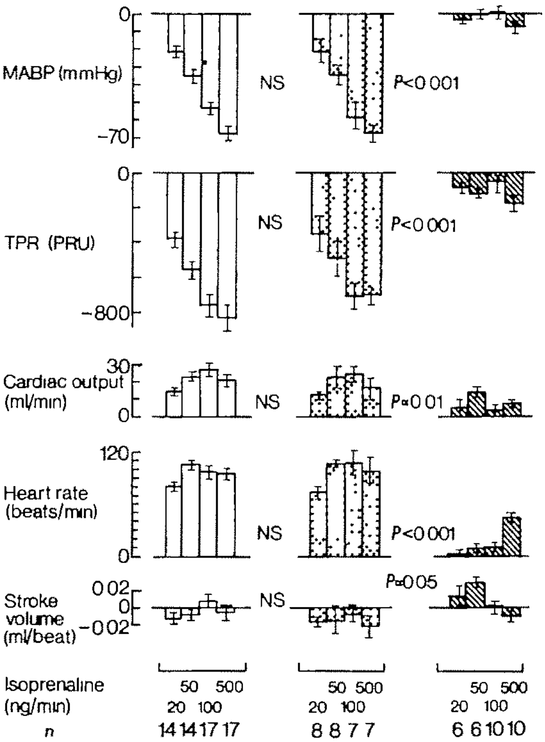

**Figure 3** Mean cardiovascular changes produced by isoprenaline infused at four rates before (open columns) and after administration of either saline (dotted column) or propranolol (hatched columns). Vertical lines show s.e. mean. The responses were compared with a chi-squared test and differences together with levels of significance are given between the groups of responses. NS signifies no difference between the groups at the 5% level of significance

### *Effects of isoprenaline infusion*

A dose-dependent fall in MABP (Figure 1b) occurred. This was due predominantly to a decrease in TPR since at all doses cardiac output was raised. The increase in cardiac output was pronounced at the lowest rate of infusion (20 ng/min); at intermediate rates (50 and 100 ng/min) cardiac output was further increased only slightly, while at the highest infusion rate (500 ng/min), output, though significantly raised above control levels, was no greater than was obtained with the lowest rate. Figure 1b clearly shows that at all rates of isoprenaline administration stroke volume was maintained and was not significantly different from

**Table 2** The cardiovascular effects of propranolol compared with those produced by saline

| | n | *MABP (mmHg)* | *TPR (PRU)* | *Cardiac output (ml/min)* | *Heart rate (beats/min)* | *Stroke volume (ml/beat)* |
|---|---|---|---|---|---|---|
| Effects of propranolol (1 mg/kg, i.v.) | 31 | −12.9±3.2 | −50±58 | −5.8±2.1 | −62±6 | +0.023±0.006 |
| Effects of saline (1 ml/kg, i.v.) | 29 | −2.3±1.5 | −48±23 | +1.5±1.4 | −9±3 | +0.011±0.003 |
| *P* | | <0.005 | NS | <0.01 | <0.001 | NS |

control levels ($P > 0.05$) despite a markedly increased heart rate.

At the four rates of isoprenaline infusion MABP fell by 21.3 ± 3.0, 35.3 ± 3.8, 53.9 ± 3.5 and 68.0 ± 4.1 mmHg (Figure 3). These reductions in MABP were produced by falls in TPR of 380 ± 42, 560 ± 49, 764 ± 61 and 839 ± 72 PRU respectively despite a cardiac output raised in each case by 14.0 ± 2.1, 23.0 ± 2.8, 26.9 ± 3.8 and 20.5 ± 3.2 ml/minute. At each infusion rate stroke volume was maintained at its resting level of approximately 0.238 ml/beat, while heart rate increased by 81 ± 4.7, 105 ± 4.5, 97 ± 7.4 and 95 ± 7.4 beats/min respectively.

### *Effects of propranolol*

In 31 of the 60 animals $\beta$-adrenoceptor blockade was produced with propranolol after the second control period. In the remaining 29 animals an equivalent volume of 153 mM NaCl solution was given instead of propranolol (Table 2).

Compared with the effects of the saline, propranolol produced a significant fall in resting heart rate. This resulted in a fall in cardiac output since stroke volume was essentially unaltered by the drug. TPR was unchanged by propranolol; MABP fell slightly due to the reduced cardiac output.

*Effects of phenylephrine after saline.* The cardiovascular responses to the four infusion rates of phenylephrine after administration of saline were in all instances similar to, and not significantly different from the responses obtained before saline (Figure 2).

*Effects of phenylephrine after propranolol.* The cardiovascular responses to the four infusion rates of phenylephrine after the administration of propranolol were compared with those responses obtained after saline (Figure 2). After $\beta$-adrenoceptor blockade phenylephrine produced a significantly greater increase in TPR than before, but in all other cardiovascular variables the responses were the same.

*Effects of isoprenaline after saline.* The cardiovascular responses to isoprenaline infused after saline administration were in no case different from those responses obtained before saline (Figure 3).

*Effects of isoprenaline after propranolol.* After propranolol administration the effects of isoprenaline on the cardiovascular system were considerably altered (Figure 3). At each of the four infusion rates the induced changes in TPR and in cardiac output were significantly smaller after propranolol than after saline administration with the result that no dose of isoprenaline now caused any significant fall in MABP. It is of interest to note that after propranolol the positive chronotropic effect of isoprenaline was significantly attenuated but the changes in stroke volume were only slightly different from those obtained before.

## Discussion

Infusion of phenylephrine into the pentobarbitone-anaesthetized rat produces a rise in MABP. This increase is brought about by both an increase in TPR and an increase in cardiac output; as the dose of phenylephrine increases the relative contribution of the raised TPR becomes greater. The increase in cardiac output is due entirely to an increase in stroke volume; heart rate remains virtually unchanged at any rate of infusion.

It is suggested that in this experimental preparation the modifying influences of the baroreceptors on the response to infused phenylephrine and isoprenaline are minimal and the measured responses therefore reflect the largely unmodified effects of these substances upon the heart and blood vessels of the rat. In support of this contention, since phenylephrine has been shown to have virtually no direct effect upon heart rate (Melville & Lu, 1952; Varma, Johnsen, Sherman & Youmans, 1960), it would appear that under the present experimental conditions baroreceptor-mediated changes in heart rate are minimal, otherwise a fall in this variable in response to the evoked rise in MABP would have been observed. In this connection Rothbaum, Shaw, Angell & Shock (1974) found that in the unanaesthetized rat, phenylephrine induced a considerable baroreceptor mediated fall in heart rate whereas De Jong & McLeod (1967) using anaesthetized rats found only a weak baroreceptor mediated fall in heart rate in response to a raised blood pressure produced by octapressin. Moreover a relatively small haemorrhage in the anaesthetized rat causes a marked fall in cardiac output and in MABP (Sapirstein, Sapirstein & Bredemeyer, 1960) suggesting again that the baroreceptor reflexes in anaesthetized rats are inadequate to cope even with relatively minor circulatory disturbance. In the present experiments an additional factor contributing to the loss of baroreceptor responses is that one of the vasosensory areas would have been non-functional because the aortic temperature sensing probe totally occluded the left common carotid artery.

Since the predominantly $\alpha$-adrenoceptor agonist phenylephrine raises TPR, it would appear that the net vasoconstriction which this indicates is mediated by $\alpha$-adrenoceptors. However, after $\beta$-adrenoceptor blockade with propranolol the increase in TPR is significantly greater, indicating a greater degree of vasoconstriction and hence that phenylephrine was previously stimulating some $\beta$-adrenoceptors which affect TPR.

The present results support the contention that the increase in cardiac output produced by infusion of phenylephrine is mediated mainly at an extracardiac location by $\alpha$-adrenoceptors. Wenzel & Su (1966) found that phenylephrine exerts a negative inotropic effect on rat ventricular strips, but a number of other investigators have described weak positive inotropic effects on the rat isolated heart (see Osnes & Øye, 1975; Rothbaum *et al.*, 1974 for references) mediated by both $\alpha$- and $\beta$-adrenoceptors, the latter being quantitatively more important (Weston, 1971). Any inotropic effect of the magnitude described by these authors would be unlikely to make a significant contribution to the raised cardiac output in response to phenylephrine infusion. It is probable therefore that in the absence of pronounced direct effects upon the heart, the increase in cardiac output is brought about by a Starling mechanism consequent upon a raised venous return which itself could be brought about by venoconstriction. This venoconstriction could be mediated by $\alpha$-adrenoceptors since $\beta$-adrenoceptor blockade does not significantly alter the response to phenylephrine.

The limitation of the increment in cardiac output at higher rates of phenylephrine infusion could be due mainly to the rise in TPR which occurs at these levels of administration. The increased resistance to blood flow from arteries to veins which results from increasing $\alpha$-adrenoceptor mediated vasoconstriction would tend to reduce venous return progressively despite any concomitant venoconstriction. In addition, the high aortic blood pressure engendered by the high TPR may itself limit the output of the heart.

Infusion of isoprenaline into the anaesthetized rat produces a fall in MABP. This is brought about entirely by a reduction in TPR, and hence by a predominance of vasodilatation, since at all levels of administration there is an increase in cardiac output. The vasodilatation is brought about mainly by the effects of isoprenaline on $\beta$-adrenoceptors since after propranolol the changes in TPR are significantly attenuated.

The increase in cardiac output which occurs during isoprenaline infusion is of interest for at each of the four infusion rates stroke volume is maintained close to its control value while heart rate is raised. The increase in heart rate is more likely to be due to direct effects of the amine upon the pacemaker than to the reduced baroreceptor activity consequent upon the fall in MABP because under our experimental conditions baroreceptor reflexes are not pronounced. Since the positive chronotropic effect is abolished by propranolol, it is mediated by $\beta$-adrenoceptors.

The evidence available does not enable us to state categorically the mechanism of the increase in cardiac output with isoprenaline. It should be noted that changes in heart rate *per se* do not necessarily influence cardiac output: if venous return remains constant, positive chronotropic responses would be compensated by inverse alterations in stroke volume. However, the reduction in stroke work (stroke volume × MABP) which we have demonstrated, combined with the positive inotropic effect that isoprenaline exerts on the rat heart (Kukovetz, Hess, Shanfeld & Haugaard, 1959; Weston, 1971) via $\beta$-adrenoceptors (Langslet, 1970; 1971) would be expected to cause a reduction in cardiac filling pressure and thereby aid the return of blood to the heart. An increase in venous return would also occur if dilatation of arteriovenous anastomoses took place. However, the most likely explanation to account for the increased cardiac output in response to isoprenaline is that the $\beta$-adrenoceptor-mediated fall in TPR induced by the amine, by reducing the resistance to blood flow from arteries to veins, enhances venous return and thus augments cardiac output. The above effects would tend to be offset by an increase in capacity of the circulation due to $\beta$-adrenoceptor mediated venodilatation which would result in pooling of blood with a consequent reduction in venous return. However, any such dilator effect which isoprenaline might have on venules and veins would appear to be relatively small because the cardiac output invariably increases in response to isoprenaline. In this connection some authors have implicated $\beta$-adrenoceptors in venoconstrictor responses in the dog (Kaiser, Ross & Braunwald, 1964) and in man (Eckstein & Hamilton, 1969). Although there is no evidence for adrenoceptors with such an action in the rat, the present results do not exclude their existence.

The cardiovascular responses obtained in the anaesthetized rat with infused phenylephrine and isoprenaline can be explained by their effects on $\alpha$- and $\beta$-adrenoceptors as classically defined. The $\alpha$-adrenoceptors are mainly extracardiac in location; the $\beta$-adrenoceptors are in the heart and elsewhere but probably do not cause substantial venodilatation in those capacitance vessels that regulate venous return to the heart.

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# RESPONSES OF THE CARDIOVASCULAR SYSTEM OF THE RAT TO NORADRENALINE INFUSIONS AND THEIR MODIFICATION BY ADRENOCEPTOR BLOCKING AGENTS

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**1** The effects of noradrenaline upon the cardiovascular system of the rat, anaesthetized with pentobarbitone, have been investigated.

**2** Noradrenaline produces a dose-dependent increase in mean arterial blood pressure (MABP) which is due entirely to an increase in cardiac output; total peripheral vascular resistance (TPR) remains unchanged.

**3** Following $\beta$-adrenoceptor blockade the pressor response to infused noradrenaline is enhanced and is now due mainly to an increase in TPR; the increment in cardiac output is reduced.

**4** After $\alpha$-adrenoceptor blockade the pressor response is greatly reduced; the residual increase in MABP is due solely to an increase in cardiac output.

**5** After ganglion blockade resting cardiac output and TPR both fall, resulting in a reduction in MABP. The pressor response to noradrenaline is enhanced and is now due to increases in both TPR and in cardiac output.

**6** The cardiovascular response of the anaesthetized rat to noradrenaline can be explained in terms of classical $\alpha$- and $\beta$-adrenoceptor stimulation by the amine; the unusual form of the response may be due to an effective predominance of $\beta$-adrenoceptor-mediated effects in this species.

**7** It is suggested that the failure of exogenous noradrenaline to produce a rise in TPR results from a balance between the $\alpha$-adrenoceptor-mediated increase and $\beta$-adrenoceptor-mediated decrease in this variable. However, this proposed balance is lost if resting vasoconstrictor tone is reduced by ganglion blockade.

## Introduction

Although it is generally recognized that intravenous administration of noradrenaline raises the arterial blood pressure in mammals, the mechanism whereby this is achieved appears to vary with the species. In man, the increase in arterial blood pressure is brought about by an increase in total peripheral vascular resistance (TPR) despite a concomitant fall in cardiac output (Barcroft & Starr, 1951; Eckstein & Abboud, 1962). Similar changes occur in the cat (Greenway & Lawson, 1966). In the dog, Levy & Brind (1957) and Shanks (1966) found that the pressor effect of noradrenaline is brought about by an increase in both TPR and in cardiac output, whereas Eckstein, Abboud & Pereda (1962) described an increase in TPR with little alteration in cardiac output. These findings suggest that the primary determinant of the pressor response to noradrenaline in these three species is an increased TPR; the changes in cardiac output being small.

In the light of these results the cardiovascular responses of the rat appear to be unusual since a change in cardiac output is the primary determinant of the pressor response to noradrenaline, the TPR being unaffected (Imms & Neame, 1974).

The present experiments were performed to elucidate the differences observed in the rat by examining the cardiovascular responses to noradrenaline after the administration of adrenoceptor and ganglionic blocking agents.

Some preliminary results have been presented to the Physiological Society (Imms, Neame & Powis, 1974).

## Methods

Thirty male rats of an SPF Wistar-derived strain and of body weight between 420 and 650 g were anaesthetized with pentobarbitone sodium ('Sagatal', May & Baker, 70 mg/kg, i.p.). Supplementary doses of the anaesthetic were given as required.

The animals were further prepared for the experiment as described in the previous paper (Imms, Neame & Powis, 1977). Heart rate and arterial blood pressure were measured and cardiac output estimated by the thermal dilution method. Total peripheral vascular resistance and stroke volume were calculated from the measured variables.

### *Infusion of noradrenaline*

Solutions of noradrenaline ((–)-noradrenaline bitartrate; 'Levophed', Winthrop) were infused through a nylon catheter inserted into the left femoral vein with a roller pump (Type MHRE7; Watson-Marlow Ltd., Falmouth, Cornwall) at flow rates between 0.12–0.15 ml/minute. The mean amounts of the catecholamine administered at each dose level were 310 ± 33 ng/min and 1480 ± 161 ng/min (mean ± s.d.).

### *Experimental procedure*

A standard protocol was adopted for all experiments. On completion of surgery, heparin (500 i.u./kg body weight, i.v.) was given and the animals were allowed to stabilize for 5 minutes.

Six control cardiac output determinations were made after which an infusion of noradrenaline at the lower rate was started. After 5 min when arterial blood pressure and heart rate were steady, six further cardiac output estimations were made. The higher dose of noradrenaline was then infused and a further series of six thermodilution curves were obtained when a steady state had been established. Noradrenaline infusion was then stopped and after 5 min, or when MABP and heart rate had returned to resting levels, six control cardiac output determinations were made.

Following this control period, either adrenoceptor or ganglionic blockade was induced. In one group of rats ($n=7$) propranolol hydrochloride (Inderal, ICI; 1 mg/kg, i.v.) was given to produce $\beta$-adrenoceptor blockade; phentolamine mesylate (Rogitine, Ciba; 2 mg/kg, i.v.) was administered to the second group of animals ($n=7$) to produce $\alpha$-adrenoceptor blockade; ganglionic blockade was produced in the third group ($n=9$) with hexamethonium (hexamethonium bromide, Sigma; 5 mg/kg, i.v. injected over 3 min) while in the fourth group of animals ($n=7$) a similar volume of 153 mM NaCl solution (1 ml/kg, i.v.) was given in place of the above drugs.

After a further 5 min for stabilization the four part schema described above was repeated. The hexamethonium-treated animals were subsequently given propranolol (1 mg/kg, i.v.) and the four part schema was again repeated.

### *Calculations*

A mean value for cardiac output during each stage of the experimental procedure was calculated from each series of six thermodilution curves and expressed in ml/minute. Over the weight range of the 30 animals used there was no relationship between resting cardiac output and mean body weight (cardiac output = 0.042 body wt. + 64.7, $r=0.114$; $P>0.05$).

Total peripheral vascular resistance was calculated as:

$$\frac{\text{MABP (mmHg)}}{\text{Cardiac output (ml/min)}} \times 10^3$$

and expressed in mmHg $\text{ml}^{-1}$ $\text{min}^{-1}\cdot 10^3$ (PRU). Stroke volume was calculated as the dividend of cardiac output and heart rate and expressed in ml/beat.

### *Responses to noradrenaline*

Absolute values for the measured or derived cardiovascular variables under control conditions are given in Table 1. The cardiovascular responses obtained in each animal with noradrenaline were calculated in terms of increments or decrements from control values, and the mean responses of the animals comprising each group were expressed as mean change ± s.e. mean. The control values from which the changes in recorded or derived cardiovascular variables were calculated were obtained by interpolation between pre-infusion and post-infusion controls.

### *Statistical analysis*

A paired $t$ test was used to evaluate the significance of the cardiovascular responses obtained with noradrenaline before adrenoceptor or ganglionic blockade (Table 1); an unpaired $t$ test was used for comparison of the response obtained after blockade with those obtained before.

## Results

### *Control data*

Before the administration of adrenoceptor or ganglionic blocking agents, the animals of all experimental groups were considered as a single population. The control cardiovascular data for these 30 animals is summarized in Table 1.

**Table 1** The cardiovascular responses to noradrenaline in the untreated rat ($n=30$)

| | *Cardiac output (ml/min)* | *MABP (mmHg)* | *TPR (PRU)* | *Heart rate (beats/min)* | *Stroke volume (ml/beat)* |
|---|---|---|---|---|---|
| Control (mean ± s d.) | 87.1 ± 19.1 | 121 ± 17 | 1475 ± 420 | 376 ± 33 | 0.235 ± 0 063 |
| Responses to noradrenaline (mean change ± s.e. mean) | | | | | |
| 300 ng/min | +18.7 ± 1.8**** | +28 ± 2**** | +25 ± 31 | +12 ± 3**** | +0.041 ± 0.005**** |
| 1500 ng/min | +32.2 ± 2.9**** | +39 ± 2**** | −12 ± 34 | +79 ± 6**** | +0.025 ± 0 007*** |
| Post-infusion control (mean ± s.d.) | 89.2 ± 19 4 | 112 ± 18 | 1327 ± 395 | 372 ± 33 | 0 240 ± 0.069 |

Statistical significance of the changes in response to noradrenaline are denoted: *** $P<0.005$; **** $P<0\ 001$)

**Table 2** The cardiovascular responses to administration of adrenoceptor or ganglion-blocking agents

| *Mean change ± s.e. mean (% change from control value)* | *Cardiac output (ml/min)* | *MABP (mmHg)* | *TPR (PRU)* | *Heart rate (beats/min)* | *Stroke volume (ml/beat)* |
|---|---|---|---|---|---|
| Effects of saline placebo ($n=7$) | −0.7 ± 6.8<br>(0.75%) | +4 ± 5<br>(3.5%) | +46 ± 78<br>(3.6%) | −3 ± 6<br>(0.83%) | −0 003 ± 0.017<br>(1 1%) |
| Effects of β-adrenoceptor blockade ($n=7$) | −0.8 ± 3.2<br>(0.93%) | −4 ± 6<br>(4 3%) | −28 ± 85<br>(2.5%) | −56 ± 6 ****<br>(15.2%) | +0.047 ± 0.014*<br>(19 9%) |
| Effects of α-adrenoceptor blockade ($n=7$) | −13.0 ± 2.2***<br>(16.0%) | −43 ± 6****<br>(40 6%) | −371 ± 91**<br>(27.9%) | +3 ± 13<br>(0.79%) | −0.031 ± 0.009*<br>(14.2%) |
| Effects of ganglion blockade ($n=9$) | −14.7 ± 4.5*<br>(14.5%) | −46 ± 5****<br>(46 9%) | −410 ± 97***<br>(39 1%) | −50 ± 9****<br>(13.9%) | −0.003 ± 0 013<br>(1 1%) |
| Effects of β-blockade after ganglion blockade ($n=9$) | +1 2 ± 5 2<br>(1.3%) | +9 ± 5<br>(13.8%) | +69 ± 22*<br>(9 2%) | −32 ± 7***<br>(9 9%) | +0 033 ± 0.020<br>(11 9%) |

Statistical significance of the changes produced by adrenoceptor or ganglion blockade are denoted: * $P<0\ 05$, ** $P<0.01$; *** $P<0.005$; **** $P<0.001$.

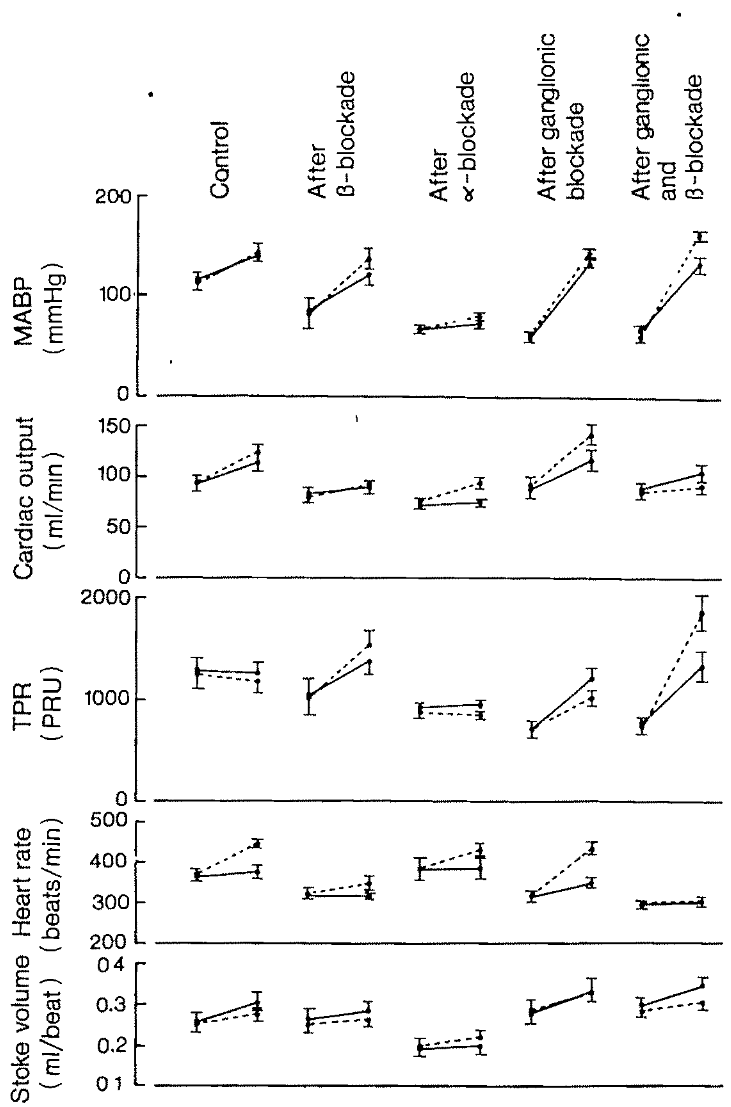

**Figure 1** The effects of intravenous noradrenaline infusion at 300 ng/min (●——●) and at 1500 ng/min (●- - -●) upon total peripheral vascular resistance (TPR), cardiac output, mean arterial blood pressure (MABP), heart rate and stroke volume in control animals, and in animals in which adrenoceptor or ganglionic blockade had been produced. The left hand filled circle of each pair shows the mean resting value of each variable and the right hand filled circle shows the mean steady state value to which this is changed by noradrenaline infusion. Vertical lines indicate s.e. mean.

In these animals both doses of noradrenaline produced significant pressor responses ($P<0.001$; Table 1). The lower rate of infusion (300 ng/min) increased the MABP by 23%; a rise that resulted from an increase in cardiac output of 21% ($P<0.001$) with no significant change in TPR (+2%; $P>0.5$). The increase in cardiac output was attributable largely to an increase in stroke volume of 18% ($P<0.001$) with a small, but significant increase in heart rate ($P<0.001$). At the higher rate of infusion (1500 ng/min) there was an increase in MABP of 32% ($P<0.001$) which resulted from an increase in cardiac output of 36% ($P<0.001$) with again no significant change in TPR. The increase in cardiac output was now due to an increase in heart rate of 21% ($P<0.001$) with an increase in stroke volume of 11% ($P<0.005$).

Administration of 153 mM NaCl solution did not cause any significant change in any of the measured cardiovascular variables (Table 2). Furthermore the cardiovascular responses to the infusions of noradrenaline were not significantly different after the saline injection from those obtained before.

The cardiovascular changes induced by noradrenaline after saline administration are shown in Figure 1 and these serve as control values with which the noradrenaline-induced cardiovascular responses obtained after adrenoceptor and ganglionic blockade can be compared.

### *β-Adrenoceptor blockade*

Administration of propranolol did not effect MABP or cardiac output but heart rate was reduced by 56 ± 6 (mean ± s.e. mean) to 312 ± 9 beats/min ($P < 0.001$), with a compensatory increase in stroke volume (Table 2).

Following propranolol administration, the responses to infusions of noradrenaline were modified. Both doses produced greater rises in MABP than those obtained before blockade and these were now due to significant increases in TPR ($P < 0.01$) with smaller increases in cardiac output ($P < 0.05$; Figure 1).

### *α-Adrenoceptor blockade*

Phentolamine administration was followed by an immediate fall in MABP of 41% ($P < 0.001$), which resulted from falls in both cardiac output (–16%; $P < 0.005$) and in TPR (–28%; $P < 0.01$, Table 2). The heart rate was unaffected but stroke volume was reduced by 0.031 ± 0.009 ml/beat (–14%; $P < 0.02$).

The pressor responses to infused noradrenaline were greatly reduced; the lower dose raised MABP by only 7 ± 3 mmHg ($P > 0.05$, Figure 1) and the larger dose produced an increase of 14 ± 3 mmHg ($P < 0.005$). The small change in MABP produced by the larger dose was due to an elevation of cardiac output alone, itself brought about by an increase in heart rate with a maintained stroke volume: there was no increase in TPR.

### *Ganglionic blockade*

Hexamethonium lowered MABP by 47% ($P < 0.001$, Table 2), a fall very similar to that produced by α-adrenoceptor blockade. The fall in MABP resulted from a decrease in TPR (–39%; $P < 0.005$) and a small fall in cardiac output (–14%; $P < 0.05$). Heart rate decreased by 50 ± 9 to 310 ± 12 beats/min, a fall of similar magnitude to that obtained following β-adrenoceptor blockade.

Pressor responses to infused noradrenaline were enhanced after ganglionic blockade; the lower dose produced a rise in MABP of 76 ± 5 mmHg ($P < 0.001$), which was due to changes in both TPR (+70%; $P < 0.001$) and cardiac output (+34%; $P < 0.02$). The higher dose of noradrenaline produced a similar pressor response (+83 ± 5 mmHg) as a result of an increase in TPR of 44% ($P < 0.001$) and an increase in cardiac output of 59% or 52.1 ± 7.2 ml/minute. The changes in heart rate observed after ganglionic blockade were 37 ± 6 beats/min during the lower rate infusion and 117 ± 12 beats/min during the higher rate of noradrenaline administration (Figure 1).

### *β-Adrenoceptor blockade after previous ganglionic blockade*

Propranolol administered to the hexamethonium-treated animal had no significant effect upon MABP or cardiac output but caused a further fall in heart rate to 292 ± 7 beats min (Table 2).

The pressor response to noradrenaline infused at 300 ng/min was similar to that obtained with ganglionic blockade alone, but the increase in cardiac output was smaller and that of the TPR was larger. The higher dose of noradrenaline increased MABP by 103 ± 4 mmHg and was now due to an increase in TPR of 170% or of 1139 ± 132 PRU. Heart rate was unchanged by noradrenaline at either dose; the increase in cardiac output obtained was due to changes in stroke volume.

## Discussion

### *Control of cardiovascular variables in the anaesthetized rat under resting conditions.*

*Heart rate.* The resting vagal tone in the anaesthetized rat is minimal (Tipton & Taylor, 1965; De Jong & McLeod, 1967). Since there is no fall in resting heart rate following α-adrenoceptor blockade it is concluded that there is no resting drive mediated by α-adrenoceptors. β-Adrenoceptor blockade however results in a considerable fall in heart rate. This could indicate either that there is a positive chronotropic drive to the heart mediated by β-adrenoceptors or, in the light of the known membrane stabilizing effect of propranolol (Langslet, 1970; 1971), that the drug is reducing the spontaneous rate of depolarization of the pacemaker cells. Since both hexamethonium, which has no reported quinidine-like action, and depletion of catecholamines from the heart (Barrett & Carter, 1970) reduce heart rate to a similar extent as propranolol, it is suggested that there is a cardioaccelerator tone maintained by a β-adrenoceptor-mediated drive.

*Vascular resistance.* The presence of α- and β-adrenoceptors affecting TPR in the rat has been demonstrated previously (Debreczeni & Fenyvesi.

1971; Imms *et al.,* 1977). Furthermore the results from α-adrenoceptor blockade indicate that there is resting vasoconstrictor tone. Since ganglion blockade reduces TPR to a similar extent to α-adrenoceptor blockade, vasoconstrictor tone would appear to be mediated by autonomic nerves. The failure of propranolol to alter resting TPR implies that there is no resting β-adrenoceptor-mediated vasodilator tone.

*Cardiac output.* We have no direct evidence for the cause of the fall in cardiac output which follows α-adrenoceptor blockade. However, it is unlikely to be due to impairment of the heart's ability to pump blood from the veins to the arteries because phentolamine has a pronounced positive inotropic effect (Gould, Zahir & Ettinger, 1969; Das & Parratt, 1971; Nayler & Carson, 1972). The most plausible explanation therefore is that the fall results from an effect of the blocking agent on the peripheral circulation rather than upon the heart. In this connection it is possible that before blockade there was an α-adrenoceptor-mediated venoconstrictor tone. Loss of such tone would result in venous blood pooling and a reduced cardiac filling pressure with a consequent fall in cardiac output.

With regard to the effects of propranolol, we found that it slowed the heart but left the cardiac output and MABP unaltered. This means that the left ventricular stroke work must have increased solely by virtue of an increase in stroke volume. Since propranolol has a negative inotropic effect on the ventricle (Shanks, 1966) this increase in stroke work must presumably have been achieved by an increase in left heart filling pressure consequent upon β-adrenoceptor blockade.

### *Noradrenaline*

In the cat and dog and in man, intravenous infusions of noradrenaline raise the MABP mainly by elevating the TPR (see introduction for references). However, the results presented above show clearly that in the rat, noradrenaline raises the MABP solely by increasing cardiac output. These findings are at variance with those of Takács (1964) who found that in the anaesthetized rat, as in other mammals, noradrenaline caused an elevation in MABP by raising TPR with no accompanying changes in cardiac output. This discrepancy may be one of technique for Takács determined cardiac output by a dye-dilution method requiring the removal of blood samples. In this connection a haemorrhage of as little as 1 ml per 100 g body weight of rat reduces cardiac output by 51% (Sapirstein, Sapirstein & Bredemeyer, 1960).

The results presented in the previous paper (Imms *et al.,* 1977) showed that in the anaesthetized rat the cardiovascular responses to catecholamines can be explained by their actions on α- and β-adrenoceptors as classically defined (Ahlquist, 1948), but it is possible that the unusual response of the rat to noradrenaline results from a different functional balance between these adrenoceptors compared to that observed in other animals. The resulting adrenoceptor-mediated effects may be modified however by compensatory baroreceptor reflexes, but such reflex re-adjustments appear to be weak in rats anaesthetized with pentobarbitone (De Jong & McLeod, 1967; Imms *et al.,* 1977). This conclusion is further supported by the present results: the elevation of heart rate which is observed during noradrenaline infusion is presumably the net result of direct cardiac pacemaker stimulation combined with reflex inhibitory effects induced by the rise in blood pressure. The stimulatory effects of noradrenaline are substantially reduced by β-adrenoceptor blockade but in no instance did such blockade result in a reflex fall in heart rate despite the fact that the increase in MABP with noradrenaline was as great or even greater after propranolol than before. Moreover, abolition of all neurally mediated autonomic efferent activity with hexamethonium failed to modify the absolute chronotropic increment produced by infused noradrenaline which suggests that before ganglion blockade autonomic reflex activity influencing this variable was not pronounced (see also De Jong & McLeod, 1967).

The present results give no indication of the extent of reflexly mediated falls in TPR which might result from the noradrenaline-induced rise in MABP. It would appear however that this reflex component is also minimal. Sapirstein *et al.* (1960) found that slight haemorrhage in the anaesthetized rat resulted in pronounced falls in cardiac output and in MABP which suggests that little baroreceptor mediated compensation took place. As indicated previously (Imms *et al.,* 1977) baroreceptor reflexes may be further reduced in the present experiments because the left common carotid artery is occluded for insertion of the aortic probe. It is suggested therefore that in the present experiments the observed cardiovascular effects of noradrenaline are due largely to the unmodified effects of the amine on α- and β-adrenoceptors.

*Heart rate.* Noradrenaline causes an increase in heart rate; this effect is mediated mainly by β-adrenoceptors since it is reduced after propranolol but is unaffected by α-adrenoceptor blockade with phentolamine.

*Vascular resistance.* The presence of α- and β-adrenoceptors in the rat which mediate changes in TPR has been demonstrated (Debreczeni & Takács, 1968; Debreczeni & Fenyvesi, 1971; Imms *et al.,* 1977). In addition it has been reported that there is a preponderance of β-adrenoceptors in the rat vasculature (Altura & Zweifach, 1965; Yamamoto &

Sekiya, 1969; 1972). It is possible therefore that when arterial baroreceptor reflexes are depressed, the absence of changes in TPR in the rat during noradrenaline infusion results from an equivalent degree of $\alpha$-adrenoceptor-mediated vasoconstriction and $\beta$-adrenoceptor-mediated vasodilatation occurring simultaneously though not necessarily in the same vascular beds. If this degree of balanced antagonism does occur, then blockade of either type of adrenoceptor should allow the unopposed effects of the other to be observed. Blockade of $\beta$-adrenoceptors allows noradrenaline to raise TPR, presumably by its effects on $\alpha$-adrenoceptors; however, blockade of $\alpha$-adrenoceptors does not uncover any $\beta$-adrenoceptor-mediated fall in TPR but in these animals the prevailing vascular tone may be too low to allow further vasodilatation to occur.

An increase in TPR is obtained with noradrenaline after ganglion blockade; this increase is enhanced by subsequent $\beta$-adrenoceptor blockade which confirms the participation of these adrenoceptors in mediating noradrenaline-induced reduction in TPR. However, if our view that there is a balance between $\alpha$- and $\beta$-adrenoceptors during noradrenaline infusion is correct, then it remains to be explained why large increases in TPR were obtained after ganglion blockade. It is suggested that when vasoconstrictor tone is reduced by hexamethonium an extra population of $\alpha$-adrenoceptors previously engaged with endogenous noradrenaline becomes available to the infused amine. A preponderance of $\alpha$-adrenoceptors available to noradrenaline thus allows the TPR to increase. Furthermore, the capacity of a vascular bed, devoid of vasoconstrictor tone, to dilate further would be reduced and any $\beta$-adrenoceptor-mediated effect must be weak. It is of interest to note that after ganglion blockade, noradrenaline infused at the lower rate raised TPR but only to a level close to the point of the proposed balance; further increase in the rate of noradrenaline infusion did not further raise TPR but in fact reduced the increment. This curtailment of the noradrenaline-induced rise in TPR is removed by subsequent $\beta$-adrenoceptor blockade which now allows a dose-dependent increase in TPR with noradrenaline (Figure 1).

*Cardiac output.* The action of noradrenaline in increasing cardiac output is probably both on the heart and on the peripheral circulation. The action on the heart is mediated by $\beta$-adrenoceptors since propranolol abolishes the positive chronotropic (these experiments) and inotropic responses (Shanks, 1966; Weston, 1971). However, propranolol does not abolish the increase in cardiac output obtained by infusion of noradrenaline. If it is assumed that propranolol completely blocked the cardiac actions of the infused noradrenaline, then the possibility remains that the residual increase in cardiac output is the result of a peripheral $\alpha$-adrenoceptor-mediated action of the catecholamine on venules and veins. In this connection $\alpha$-adrenoceptor blockade alone was found to diminish, but not abolish the noradrenaline induced increase in cardiac output.

The results of the crucial experiment of testing the effect of noradrenaline after combined $\alpha$- and $\beta$-adrenoceptor blockade were equivocal because of the inability of the preparation to tolerate the combination of the blocking agents used.

Although the use of $\alpha$- and $\beta$-adrenoceptor blocking agents has helped to elucidate the mechanisms of action of noradrenaline in terms of its cardiac versus peripheral actions, further studies are required to evaluate quantitatively the relative contribution of its actions on capacitance vessels to the overall response.

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# A COMPARISON OF THE EFFECTS OF BRADYKININ, 5-HYDROXYTRYPTAMINE AND HISTAMINE ON THE HEPATIC ARTERIAL AND PORTAL VENOUS VASCULAR BEDS OF THE DOG: HISTAMINE $H_1$ AND $H_2$-RECEPTOR POPULATIONS

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1 The hepatic arterial and hepatic portal venous vascular beds of anaesthetized dogs were separately perfused in different experiments.

2 From measurements of perfusion pressures and blood flows in the two series of experiments, hepatic arterial vascular resistance (HAVR) and hepatic portal venous vascular resistance (HPVR) respectively were calculated.

3 Bradykinin, 5-hydroxytryptamine (5-HT) and histamine were injected intra-arterially and intraportally and dose-response curves constructed from these data.

4 Bradykinin injected intra-arterially caused dose-dependent hepatic arterial vasodilatation, and with an $ED_{50}$ of $2.66 \times 10^{-13}$ mol was more potent than any other vasodilator agent yet examined on this vascular bed.

5 Bradykinin injected intraportally at doses up to 10 times those which were maximal on the arterial circuit did not alter the calculated HPVR.

6 5-HT injected intra-arterially caused weak and variable rises in HAVR, indicating vasoconstriction. The maximum rise in HAVR was much less than that attained with noradrenaline in the same preparations.

7 5-HT injected intraportally caused dose-dependent rises in HPVR indicating portal constriction at doses above 15–100 µg: in some experiments small doses of 5-HT resulted in reductions in calculated HPVR.

8 Histamine has previously been shown to cause hepatic arterial vasodilatation: by intraportal injection, it caused dose-dependent rises in HPVR.

9 In order to examine the receptors responsible for the effects of histamine, dose-response curves were constructed before and after mepyramine and metiamide.

10 On the hepatic arterial vascular bed, metiamide did not antagonize the vasodilator effects of intra-arterial histamine, but these effects were antagonized by mepyramine.

11 Similarly on the hepatic portal bed, the rises in HPVR due to histamine were antagonized by mepyramine but not by metiamide.

12 The effects of histamine on both the hepatic arterial and portal venous vascular beds of the dog are therefore mediated predominantly by histamine $H_1$-receptors.

## Introduction

Injections of noradrenaline and angiotensin into the hepatic artery of the anaesthetized dog cause dose-dependent hepatic arterial vasoconstriction (Andrews, Hecker, Maegraith & Ritchie, 1955; Richardson & Withrington, 1976a); when administered by injection into the hepatic portal vein they cause portal venous vasoconstriction although the responses to angiotensin show marked tachyphylaxis (Richardson & Withrington, 1977). Vasopressin, whilst causing profound dose-dependent vasoconstriction of the hepatic arterial vascular bed (Richardson & Withrington, 1976a) has little effect on the resistance sites of the portal system except with very high doses when it causes a reduction in portal vascular resistance (Richardson & Withrington, 1977). Quantitative and qualitative differences in the responses of the hepatic arterial and portal vascular beds to naturally-occurring substances are therefore evident.

Bradykinin, 5-hydroxytryptamine (5-HT) and histamine all occur naturally, and whilst not normally present in the systemic circulation in vasoactive amounts, they may enter the liver in the portal vein having been released from the intestine, pancreas or spleen. In addition, they may be synthesized within the liver and thereby reach the sites controlling the hepatic arterial and portal vascular resistance. Furthermore, in various pathological states (e.g. anaphylaxis, shock) they may be released from the gut in such quantities as to enter the systemic circulation in vasoactive amounts. In such circumstances therefore, these substances may enter the liver by both the hepatic arterial and portal venous routes.

In the present experiments bradykinin, 5-HT and histamine were administered in increasing graded doses into the hepatic arterial and hepatic portal venous beds when these were separately perfused, in different experiments. The possible physiological implications of the vascular responses to these substances at both sites in the control and regulation of total liver blood flow are discussed.

In addition, by the use of the selective histamine $H_1$ and $H_2$-receptor antagonists, mepyramine and metiamide (Black, Duncan, Durant, Ganellin & Parsons, 1972; Black, Owen & Parsons, 1975), the nature of the receptors responsible for the effects of histamine has been examined.

## Methods

Experiments were performed on a total of 17 dogs, weighing between 9.6 and 20.9 kg, which had been deprived of food, but allowed free access to water for 24 h before the induction of anaesthesia by an intravenous injection of methohexitone sodium (Brietal, Lilly: 7.5–12.5 mg/kg). Anaesthesia was maintained with chloralose (Kuhlmann, Paris: 50 mg/kg) and urethane (BDH: 500 mg/kg), intravenously, supplements of chloralose and urethane in the same proportions being given as necessary throughout the experiments to maintain a constant level of anaesthesia.

Two types of preparation were used: one ($n = 7$) in which the common hepatic artery was perfused from a femoral artery and the hepatic arterial blood flow and perfusion pressure were monitored ('arterial preparations'), and the other ($n = 10$) in which the hepatic portal vein was perfused at constant flow from a reservoir into which drained blood from the superior mesenteric vein; in these preparations ('portal preparations'), the hepatic portal vein perfusion pressure was monitored continuously. These preparations have been described previously (arterial: Richardson & Withrington, 1976b; portal: Richardson & Withrington, 1977) and only brief details of the surgical preparation are given here.

In all experiments, possible systemic effects of large doses of vasoactive agents injected locally to the liver were assessed by measurement of the systemic arterial blood pressure and heart rate. Systemic arterial blood pressure was measured from a cannulated femoral artery with a Statham P23Gb strain gauge transducer; phasic pressure was recorded throughout all experiments, and in some experiments, mean pressure derived with an averaging circuit with selectable time constants (Devices, model 3502). Heart rate was measured with a ratemeter (Devices, model 4521) triggered from the pulsatile systemic arterial pressure waveform.

The inferior vena caval pressure (IVCP) was measured at the level of the hepatic vein from a catheter advanced for a known distance through a cannulated femoral vein, using a Consolidated Electrodynamics L212 strain gauge transducer. The position of the catheter tip was confirmed *post mortem*.

All animals received 250 iu/kg heparin (Weddel Pharmaceuticals) immediately before cannulation of the blood vessels, and supplements of 100 iu/kg were given hourly. All cannula systems were primed with a solution of 10% low molecular weight dextran in normal saline (Rheomacrodex, Pharmacia).

### *Arterial preparations*

Following a midline laparotomy, the common hepatic artery was separated from its periarterial nerves which were carefully preserved. The hepatic artery was cannulated and perfused from a femoral artery. The blood flow in this cannula system (hepatic arterial blood flow; HABF) was measured with a cannulating flow probe and Cardiovascular Instruments square-wave pulsed-field electromagnetic flowmeter (model 3765T), and the hepatic arterial perfusion pressure (PP) was measured from a 'T' piece in the cannula system close to the point of cannulation of the hepatic artery, using a Consolidated Electrodynamics L212 strain gauge transducer.

Hepatic arterial mean blood flow was derived by passing the phasic signal through an averaging circuit with a time constant of 0.6 second. Hepatic arterial mean perfusion pressure was derived by passing the phasic signal through an averaging circuit (Devices, model 3502) with selectable time constants of 0.5, 1 and 2 seconds. Both mean and phasic flow and pressure records were displayed continuously.

### *Portal preparations*

After a midline laparotomy, the hepatic portal, superior mesenteric and splenic veins were cleared from surrounding tissue. The splenic artery was tied and the splenic nerves stimulated supramaximally to expel stored erythrocytes into the circulation; the splenic vein was then tied and cannulated retrogradely, and the hepatic portal vein tied about 5 mm from the confluence of the superior mesenteric and splenic veins: in this way, the outflow from the superior mesenteric vein was diverted via the cannulated splenic vein into a small reservoir. The portal vein was cannulated towards the liver, and perfused with blood from the reservoir by means of a roller pump (Watson Marlow, MHRE-200). Hepatic portal venous perfusion pressure (HPVP) was recorded from a 'T' piece close to the point of cannulation of the portal vein with a Statham P23V strain gauge transducer. The hepatic portal inflow was monitored with a cannulating flowprobe on the outflow side of the roller pump, and a Cardiovascular Instruments electromagnetic flowmeter. Mean pressures and flows were derived as in the arterial preparations.

### *Calibrations and recordings*

All pressure transducers were calibrated with mercury or water manometers before each experiment; zero positions were taken as those recorded with the catheter tips exposed to air *in situ, post mortem.* Cannulating flow probes were calibrated *in situ* with whole blood at the end of each experiment; occlusive flow zeros were established frequently throughout each experiment, in the portal preparations by diverting the blood flow through a bypass in parallel with the flow probe.

After appropriate amplification, all variables were recorded continuously on a Devices M-19 rectilinear recorder.

### *Calculations*

*Liver weight* was obtained *post mortem* in every experiment; values expressed per 100 g refer to this terminal weight of liver.

*Hepatic arterial vascular resistance* was calculated as (hepatic arterial mean perfusion pressure: mmHg)/(hepatic arterial mean blood flow: ml/min, or ml $\text{min}^{-1}$ 100 $\text{g}^{-1}$), and expressed as mmHg $\text{ml}^{-1}$ min, or mmHg $\text{ml}^{-1}$ min 100 g.

*Hepatic portal vascular resistance* was calculated as (hepatic portal mean perfusion pressure – inferior vena cava pressure: mmHg)/(hepatic portal mean blood flow: ml/min or ml $\text{min}^{-1}$ 100 $\text{g}^{-1}$) and expressed in the same units as the hepatic arterial vascular resistance.

*Changes in vascular resistance* were always calculated as percentage changes from control values to the peak of the effect of the vasoactive substance, i.e. (change in vascular resistance × 100)/(control vascular resistance).

### *Expression of results*

Initial control values are expressed as means ± 1 s.d., and all other values are expressed as means ± s.e. means. The $\text{ED}_{50}$ values represent the doses of substances which would produce 50% of the maximum effect attained by progressively increasing administrations of those substances.

In the arterial preparations, the arterial perfusion pressure normally remained constant, and therefore changes in calculated hepatic arterial vascular resistance (HAVR) indicate hepatic arterial vasoconstriction or vasodilatation. However, the injection of large doses of the vasoactive agents led to systemic vascular effects manifest as small changes in systemic arterial mean blood pressure; these were reflected in equivalent small changes in hepatic arterial perfusion pressure accompanying the large drug-induced changes in hepatic arterial blood flow. The myogenic and hydrostatic changes in vascular resistance arising from these changes in intravascular pressure *per se* (Bayliss, 1902; Folkow, 1964) are, in these preparations, negligible compared with the drug-induced changes in HAVR, whether vasoconstrictor (Richardson & Withrington, 1976a) or vasodilator (Richardson & Withrington, 1976b) drugs are administered: consequently, no correction factors have been applied for changes in HAVR resulting from alterations in perfusion pressure *per se.*

The portal preparations were perfused at constant flow, and the inferior vena caval pressure remained constant throughout (see Results section), so that changes in calculated hepatic portal vascular resistance (HPVR) indicate hepatic portal vasoconstriction or vasodilatation.

### *Drugs*

The drugs used were: bradykinin triacetate (Sigma; mol. wt. = 1420.5), 5-hydroxytryptamine creatinine sulphate (5-HT, BDH; mol. wt. = 307.1), mepyramine maleate (M & B; mol. wt. = 401.5), metiamide (SK & F; mol. wt. = 244.4) and noradrenaline acid tartrate (Levophed, Winthrop; mol. wt. of base = 169.0). Doses are expressed in terms of the salts used except for noradrenaline where doses are expressed in terms of the base; molar concentrations were calculated from the molecular weights shown above, which are derived from the manufacturers' data. Bradykinin, 5-HT, histamine and mepyramine were dissolved in 0.9% w/v NaCl solution (saline), and metiamide solutions prepared as described by Black *et al.* (1975). All solutions were prepared fresh for each experiment.

Agonist drugs were injected directly into the cannula system, either intra-arterially (i.a.) or into the portal vein (intraportally). To construct dose-response curves, increasing doses of agonists were given, separated by at least 1 min from complete recovery from the effects of the preceding dose. Antagonists were administered intravenously (i.v.), mepyramine by injection, but metiamide, because of its comparatively short plasma half-life in the dog of 40–60 min (Black *et al.*, 1975; manufacturer's data), was infused intravenously for at least 10 min before any subsequent histamine administrations, and the infusion was continued throughout the period of the subsequent histamine administrations.

## Results

### *Control values*

*(a) Hepatic arterial preparations.* Seven dogs weighing between 10.3 and 15.3 kg (12.4 ± 3.2 kg) were used in these experiments; *post mortem*, the livers weighed 288.9 ± 54.2 grams. Under control conditions, the systemic arterial mean blood pressure was 126.3 ± 13.4 mmHg and the inferior vena caval pressure (IVCP) 1.8 ± 0.5 mmHg. The hepatic arterial blood flow was 219.1 ± 49.9 ml/min, or 77.1 ± 20.3 ml $min^{-1}$ 100 $g^{-1}$; the hepatic arterial mean perfusion pressure (PP) was 114.9 ± 14.2 mmHg, giving a calculated hepatic arterial vascular resistance (HAVR) of 0.55 ± 0.13 mmHg $ml^{-1}$ min, or 1.55 ± 0.34 mmHg $ml^{-1}$ min 100 grams. These values are similar to those found previously for the sympathetically-innervated hepatic arterial vascular bed (Richardson & Withrington, 1976b,c,d).

*(b) Hepatic portal preparations.* The 10 dogs used in these investigations weighed between 9.6 and 20.9 kg (16.0 ± 4.3 kg), the livers weighing 328.1 ± 80.1 grams. Under control conditions, the systemic arterial mean pressure was 135.5 ± 13.0 mmHg and the inferior vena caval pressure 1.7 ± 0.98 mmHg. The hepatic portal inflow was 259.1 ± 83.7 ml/min or 78.7 ± 13.0 ml $min^{-1}$ 100 $g^{-1}$, and at this inflow the hepatic portal perfusion pressure was 5.6 ± 1.86 mmHg. The calculated hepatic portal venous vascular resistance was 0.016 ± 0.007 mmHg $ml^{-1}$ min, or 0.056 ± 0.022 mmHg $ml^{-1}$ min 100 grams. These values are similar to those reported previously for this preparation from laboratory (Richardson & Withrington, 1977).

### *Effects of injections of bradykinin*

*(a) Hepatic arterial system.* Bradykinin was injected into the hepatic artery over the dose range 10 pg to 100 ng on 6 occasions in 3 preparations. The only effect observed with doses above threshold was an increase in hepatic arterial blood flow of short duration. This response was graded and increased with increasing doses; very high doses of bradykinin were accompanied by small reductions in perfusion pressure. These increases in hepatic arterial blood flow represent dose-dependent reductions in calculated hepatic arterial vascular resistance and signify hepatic arterial vasodilatation.

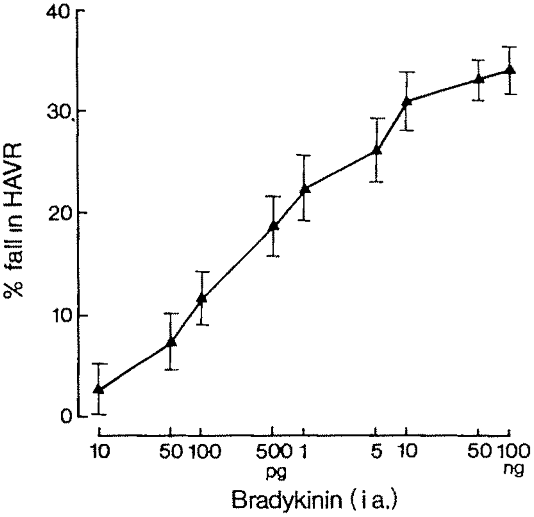

**Figure 1** $Log_{10}$ dose-response curve for bradykinin on the hepatic arterial vascular bed of the dog. The dose is expressed in terms of the weight of the salt injected intra-arterially (abscissa scale) and the response as the percentage fall in the calculated hepatic arterial vascular resistance (HAVR) (ordinate scale). The points represent the means of 6 observations and the vertical bars show the s.e. means.

The threshold dose of bradykinin when injected i.a. was either 10 or 50 pg, and the maximum reduction in calculated hepatic arterial resistance which occurred on injection of either 50 or 100 ng was 34.4 ± 2.4%; a value similar to the maximum reduction in hepatic arterial vascular resistance reported previously (Richardson & Withrington, 1976b) for the intra-arterial injection of a series of other vasodilator substances.

The $log_{10}$ dose-response curve for intra-arterial injection of bradykinin on the hepatic arterial vascular bed is shown in Figure 1; the mean $ED_{50}$ is 330 pg or 2.66 x $10^{-13}$ mol. The effects of maximal vasodilator doses of bradykinin on the hepatic arterial blood flow, hepatic arterial perfusion pressure and the calculated hepatic arterial vascular resistance are shown in Table 1.

*(b) Hepatic portal venous vascular system.* Bradykinin was injected intraportally in

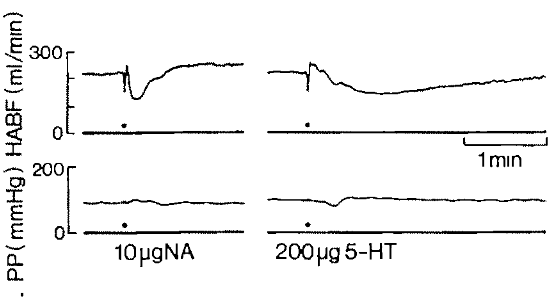

**Figure 2** Time courses of the responses of the hepatic arterial vascular bed to intra-arterial injections of 5-hydroxytryptamine (5-HT) and noradrenaline (NA). HABF=hepatic arterial blood flow, PP=hepatic arterial mean perfusion pressure.

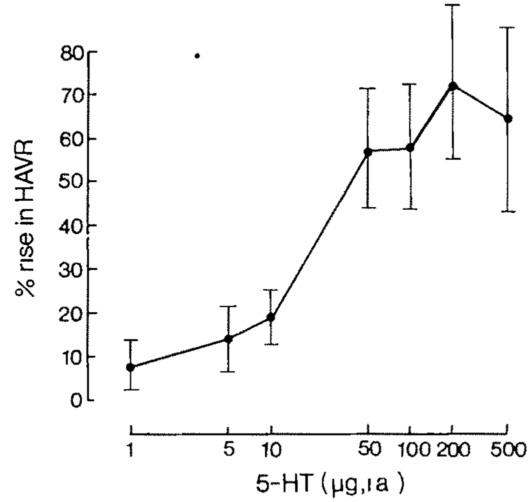

**Figure 3** $\text{Log}_{10}$ dose-response curve for 5-hydroxytryptamine (5-HT) on the hepatic arterial vascular bed of the dog. The dose is expressed in terms of the weight of the salt injected intra-arterially (abscissa scale), and the response as the percentage rise in the calculated hepatic arterial vascular resistance (HAVR) (ordinate scale). The points represent the means of 5 observations, and the vertical bars show the s.e. means.

doses between 1.0 ng and 10 µg once in each of 3 experiments. No detectable change in hepatic portal perfusion pressure resulted from any of these injections (Figure 4); the hepatic portal blood flow and IVCP remained constant. There was, therefore, no change in the calculated portal vascular resistance to any of the doses of bradykinin used in this series. Doses in excess of 500 ng caused reductions in systemic arterial pressure, presumably due, at least in part, to systemic vasodilatation; these observations confirmed the biological activity of the solution used.

*Effects of injections of 5-hydroxytryptamine*

*(a) Hepatic arterial system.* 5-HT was injected intra-arterially over the dose range 500 ng to 500 µg to construct 6 dose-response curves in 5 preparations: at doses above the threshold, there were usually decreases in hepatic arterial blood flow of long duration. The hepatic arterial perfusion pressure either remained constant or increased slightly with the higher doses of 5-HT, and consequently the reductions in hepatic arterial blood flow represent increases in the calculated hepatic arterial vascular resistance, and hepatic arterial vasoconstriction. The threshold varied between 0.5 and 10 µg in different preparations, and the maximum rise in calculated HAVR occurred at doses between 50 and 500 µg: a typical response is shown in Figure 2 and the results are summarized in Figure 3. The effects of maximal vasoconstrictor doses of 5-HT on hepatic arterial perfusion pressure, blood flow, and calculated HAVR are shown in Table 1.

In 2 experiments there was an apparent small

**Table 1** Hepatic arterial perfusion pressure (PP), blood flow (HABF) and calculated hepatic arterial vascular resistance (HAVR) of the dog immediately before, and at the peak of responses to maximal doses of bradykinin and 5-hydroxytryptamine

| *PP (mmHg)* | | *HABF (ml min$^{-1}$ 100 g$^{-1}$)* | | *HAVR (mmHg ml$^{-1}$ min 100 g)* | |
|---|---|---|---|---|---|
| *Control* | *Peak* | *Control* | *Peak* | *Control* | *Peak* |
| *Bradykinin (n=6)* | | | | | |
| 111.8±4.2 | 104.3±4.0 | 56.0±4.6 | 79.6±5.6 | 2.06±0.18 | 1.35±0.13 |
| *5-hydroxytryptamine (n=5)* | | | | | |
| 94.6±6.7 | 97.2±8.1 | 76.1±11.1 | 45.0±8.5 | 1.38±0.27 | 2.57±0.64 |

Each value is the mean±s.e. mean immediately before (Control) and at the peak of the responses to maximally effective doses of the two substances (Peak). The number of dose-response curves from which the data were derived is shown in parentheses.

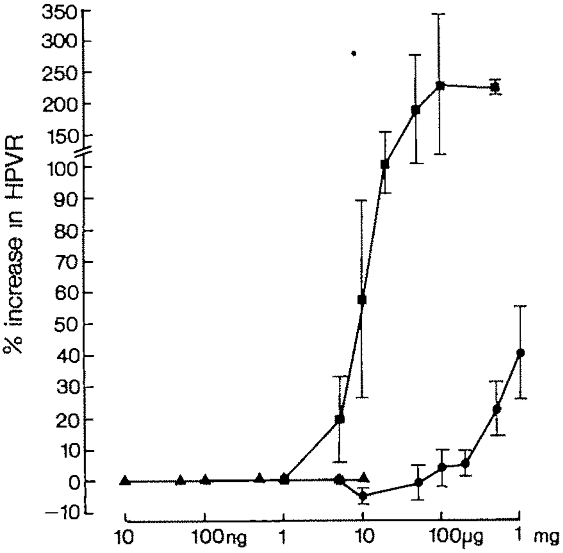

**Figure 4** $Log_{10}$ dose-response curves for bradykinin (▲), histamine (■) and 5-hydroxytryptamine (●) on the hepatic portal venous vascular bed of the dog. The doses are expressed in terms of the weight of each substance injected intraportally, and the response as the percentage rise in the calculated hepatic portal vascular resistance (HPVR). The symbols represent the mean observations (for bradykinin, $n=3$; for histamine, $n=5$; for 5-HT, $n=5$), and the vertical bars show the s.e. means.

transient increase in hepatic arterial blood flow prior to the main dose-dependent decrease; this initial increase in blood flow was not clearly separable from the injection artifact, and was not dose-dependent.

Hepatic arterial vasoconstriction to 5-HT was weak, and variable between experiments: the maximum increase in HAVR of 80.6 ± 13.7% was much smaller than the maximum increase (365.4 ± 120.9%) in HAVR to noradrenaline in the same preparations. The form and time-course of responses to 5-HT also differed from that of other vasoconstrictor agents in that the effect was of slow onset and long duration compared with that of noradrenaline (Figure 2), adrenaline, angiotensin and vasopressin (Richardson & Withrington, 1976a & unpublished observations).

*(b) Hepatic portal venous vascular system.* 5-HT was injected into the portal vein in doses from 1.0 µg to 1.0 mg on one occasion in each of 6 preparations. In 4/6 preparations, the lower doses within this range caused very small decreases in calculated hepatic portal vascular resistance (HPVR) and higher doses caused increases in HPVR. In the other 2 preparations, only increases in HPVR were seen with doses above threshold. Over the 6 experiments, the lowest dose to cause an increase in hepatic portal vascular resistance was either 50 or 100 µg. The mean effects from all six preparations are shown in Figure 4 which reveals a dose-dependent effect of reductions in HPVR at low doses and increases in HPVR at high doses of 5-HT. The time course of the effects was similar to that of other agents which increase the hepatic portal vascular resistance (Richardson & Withrington, 1977).

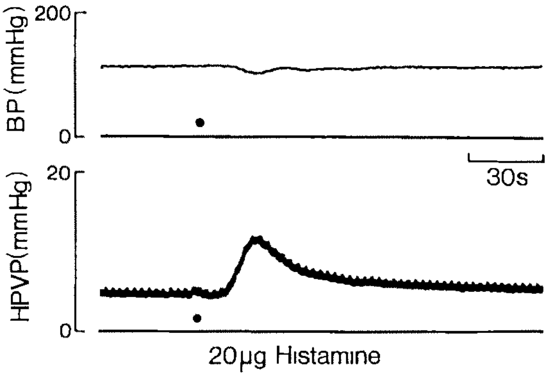

**Figure 5** Response of the hepatic portal vascular bed to an intraportal injection of histamine BP = mean systemic arterial blood pressure. HPVP = hepatic portal venous perfusion pressure. Point of injection shown by circles. Portal venous vascular bed perfused at constant blood flow (264 ml/min) and constant inferior vena caval pressure (1.5 mmHg)

*Effects of injections of histamine*

*(a) Hepatic arterial system.* By intra-arterial injection, histamine causes dose-dependent reductions in the calculated hepatic arterial vascular resistance (Figure 6); the time course of these effects and relative molar potency of histamine compared with other vasodilator agents have been published previously (Richardson & Withrington, 1976b).

*(b) Hepatic portal venous vascular system.* Intraportal injections of between 1 and 500 µg of histamine were made once in each of 5 preparations in the absence of antagonists. All doses of histamine above the threshold caused rises in hepatic portal perfusion pressure, which at constant flow and IVCP represent increases in calculated hepatic portal vascular resistance. These effects were dose-dependent, and the effects of the smaller doses were without significant systemic effects though at higher doses, reductions in systemic arterial blood pressure and rises in heart rate occurred due to histamine passing through the liver into the systemic circulation in vasoactive amounts: in these cases, the changes in portal perfusion pressure

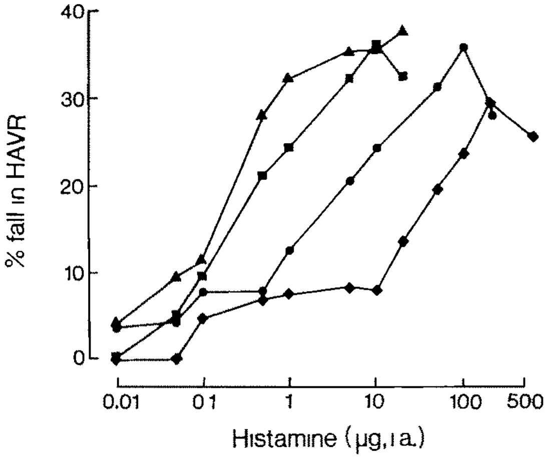

**Figure 6** $Log_{10}$ dose-response curves for histamine on the hepatic arterial vascular bed of the dog. The doses are expressed in terms of the weight of the salt injected intra-arterially, and the responses as the percentage fall in calculated hepatic arterial vascular resistance (HAVR). (■) Effects of histamine alone, in the absence of antagonists; (▲) during the infusion of $2.0 \times 10^{-6}$ mol $kg^{-1}$ $min^{-1}$ metiamide, i.v.; (●) after mepyramine, $2.5 \times 10^{-6}$ mol/kg, i.v.; (◆) after mepyramine, $5.0 \times 10^{-6}$ mol/kg, i v.

preceded the systemic effects (Figure 5). The complete dose-response curve is shown in Figure 4.

### *Histamine receptors in the canine liver vasculature*

Dose-response curves to locally administered histamine were constructed in separate arterial and portal preparations in the absence of antagonists and subsequently both during metiamide infusions and after injections of mepyramine. The antagonists were administered in both orders in different preparations, the doses being selected from the observations of Black *et al.* (1975) which preliminary experiments showed to be effective.

*(a) Hepatic arterial system.* In one experiment, the intravenous infusion of metiamide, $2.0 \times 10^{-6}$ mol $kg^{-1}$ $min^{-1}$, which was without effect on the calculated hepatic arterial vascular resistance did not antagonize the effects of i.a. histamine on this vascular bed; indeed, during the metiamide infusion, there was a slight shift of the histamine dose-response curve to the left, with no effect on the maximum response (Figure 6). Mepyramine was then injected in a dose of $2.5 \times 10^{-6}$ mol/kg (1 mg/kg) i.v., similarly resulting in no change in the calculated HAVR, but the subsequent dose-response curve to histamine showed a marked parallel shift to the right without suppression of the maximum response. A second i.v. injection of

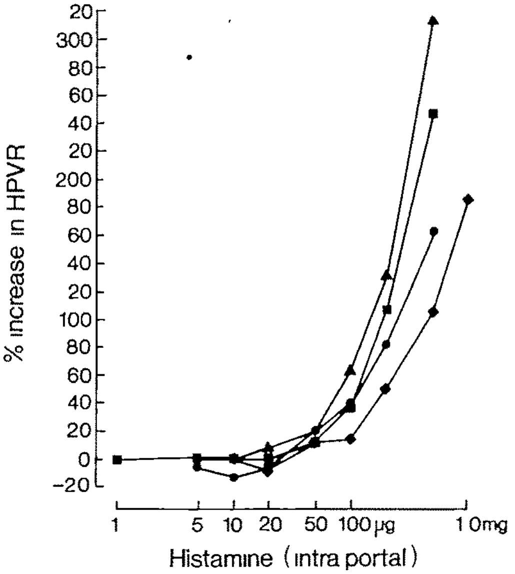

**Figure 7** $Log_{10}$ dose-response curves for histamine on the hepatic portal venous vascular bed of the dog. The doses are expressed in terms of the weight of the salt injected intraportally, and the responses as the percentage rise in the calculated hepatic portal venous vascular resistance (HPVR). Symbols and doses of antagonists as in Figure 6.

the same dose of mepyramine caused a further shift of the histamine dose-response curve to the right, though at this dose of mepyramine (total $5.0 \times 10^{-6}$ mol/kg, i.v.), there was some suppression of the maximum response to histamine (Figure 6).

Paired analysis of the effects of the various doses of histamine injected in this procedure revealed that, over the whole dose ranges used, the increased effect of histamine after metiamide ($P < 0.005$), and the reduced effects of histamine after $2.5 \times 10^{-6}$ mol/kg mepyramine ($P < 0.05$) and $5.0 \times 10^{-6}$ mol/kg mepyramine ($P < 0.02$) were all statistically significant. In contrast, in the absence of antagonists, variations in the form of position of the dose-response curves to histamine in individual experiments were very small, and identical analysis to that used above revealed that the differences between dose-response curves with the same time interval were not statistically significant ($P > 0.70$; Richardson & Withrington, unpublished observations).

In another experiment, mepyramine $2.5 \times 10^{-6}$ mol/kg was injected i.v. between the construction of two histamine dose-response curves, causing a marked parallel shift of the histamine dose-response curve to the right without suppression of the maximum response. The $ED_{50}$ before mepyramine was $4.6 \times 10^{-9}$ mol (1.4 µg) and after mepyramine was increased more than tenfold to $6.5 \times 10^{-8}$ mol (20.0 μg). In the presence of mepyramine, a subsequent infusion of metiamide ($2.0 \times 10^{-6}$ mol kg$^{-1}$ min$^{-1}$, i.v.) caused no further antagonism of the effects of histamine; indeed, there was a small parallel shift of the histamine dose-response curve to the left ($ED_{50} = 3.3 \times 10^{-8}$ mol or 10.1 μg). Paired analysis of the effects of the various doses of histamine used in this procedure revealed that the antagonism by mepyramine significantly attenuated the vasodilator effects of histamine ($P < 0.005$), but that the subsequent effect of metiamide did not significantly affect the responses to histamine ($P > 0.05$).

*(b) Hepatic portal venous vascular system.* Identical procedures to those used in the arterial preparations were repeated for the portal preparations. Metiamide, $2.0 \times 10^{-6}$ mol kg$^{-1}$ min$^{-1}$, i.v., caused a small parallel shift of the histamine dose-response curve to the left, whilst subsequent injections of mepyramine i.v. to total doses of 2.5 and $5.0 \times 10^{-6}$ mol/kg resulted in successive parallel shifts of the histamine dose-response curve to the right (Figure 7). In a further experiment, mepyramine in the absence of metiamide caused a parallel shift of the histamine dose-response curve to the right, and a subsequent infusion of metiamide ($2.0 \times 10^{-6}$ mol kg$^{-1}$ min$^{-1}$, i.v.) caused no further shift to the right of the histamine dose-response curve.

The effects of histamine in causing reductions in hepatic arterial vascular resistance and increases in hepatic portal venous vascular resistance are therefore attenuated by mepyramine but not by metiamide.

## Discussion

The two preparations used in the present series of experiments were identical to those described previously from this laboratory for the investigation of the responses of both the hepatic arterial and hepatic portal venous vascular beds of the dog to injections of a range of vasoactive compounds (Richardson & Withrington, 1976b,c; 1977). The control values for hepatic arterial and hepatic portal vascular resistances agree well with those figures previously reported. In the present paper the effects of three naturally-occurring autacoids, bradykinin, 5-HT and histamine, administered in different preparations to the hepatic arterial and portal vascular territories were examined.

All intra-arterial doses of bradykinin above the threshold caused a dose-dependent reduction in hepatic arterial vascular resistance. On a molar basis, it was the most potent dilator of the hepatic arterial vascular bed yet examined; the $ED_{50}$ was $2.66 \times 10^{-13}$ mol compared with $2.62 \times 10^{-11}$ and $5.82 \times 10^{-11}$ mol for secretin and prostaglandin $E_2$ respectively which were the most potent agents previously examined (Richardson & Withrington, 1976b). In contrast to its action on the arterial bed, bradykinin was without any direct action on the hepatic portal vascular bed even at a dose 100 times that which was maximal on the arterial system. These experiments confirm and extend the previous observations of Scholtholt & Shiraishi (1968) that intra-arterial or intraportal infusions of bradykinin cause increases in canine hepatic arterial blood flow, but are without measurable effect on the portal venous vascular bed.

Under normal circumstances, bradykinin is unlikely to attain vasoactive levels in the systemic arterial circulation because of its rapid inactivation in the lungs (Vane, 1969) and blood (Douglas, 1975). However, its synthesis and release (Seki, Nakajima & Erdös, 1972) is suspected to occur in many pathological conditions including gastrointestinal tract disorders such as the carcinoid and dumping syndromes, septic shock, pancreatitis and may explain some of the symptoms following regional hypotension consequent to occlusion of the superior mesenteric artery (Berry, Collier & Vane, 1970; Haglund, Hultén, Åhren & Lundgren, 1975; Lundgren, Hagland, Isaksson & Abe, 1976).

Under these circumstances, with high circulating levels of the polypeptide, both hepatic inflow circuits would be influenced, since in addition to hepatic arterial vasodilatation, bradykinin could increase intestinal (Fasth & Hultén, 1973), gastric (Fasth & Martinson, 1973) and splenic (Moerman, Scapagini & de Schaepdryver, 1969; B.N. Davies & P.G. Withrington, unpublished observations) blood flow resulting in a substantial increase in the inflow into the portal vein.

The bulk of the body's store of 5-HT is in the enterochromaffin and related cells of the gastrointestinal tract (Erspamer, 1954; Gabella & Juorio, 1973; Douglas, 1975) and it is known that the dog small intestine releases 5-HT into the portal vein in response to a range of injurious stimuli (Zweifach, 1962; Burks & Long, 1966). Pathologically, 5-HT together with other hormones, may enter the portal vein, and therefore the liver, from secreting tumours of the pancreas and gastro-intestinal tract (Janoff, Nagler, Baez & Zweifach, 1961; Owman, Hakanson & Sundler, 1973; Murray-Lyon, Rake, Marshall & Williams, 1973); the avidity with which the lungs take up 5-HT is so great that it is unlikely that any enters the systemic circulation from such sources in amounts to cause cardiovascular effects. In addition, amine-secreting tumours are found outside the gastro-intestinal tract and 5-HT may be liberated in large amounts into the general circulation. The present experiments establish the complete dose-response relationship for 5-HT on the hepatic arterial vascular bed and demonstrate that the amine is weakly vasoconstrictor. Similarly the predominant action of 5-HT on the portal vascular bed was weakly vasoconstrictor; but the lower doses (< 100 μg) in some experiments caused a reduction in the portal vascular resistance. 5-HT was considerably less potent in increasing portal vascular resistance than adrenaline, noradrenaline (Richardson & Withrington, 1977) or histamine but more potent than bradykinin. Confirming previous observations (Andrews & Butterworth, 1958), the responses to 5-HT of both the arterial and portal beds were more variable than those to any substance hitherto investigated. This variability in response to 5-HT may depend upon the prevailing sympathetic vasoconstrictor tone (Page, 1957; Haddy, Gordon & Emmanuel, 1959; McCubbin, Kaneko & Page, 1962; Davies & Withrington, 1973) and the route by which the amine is administered (Biber, Fara & Lundgren, 1973; Richardson, 1974).

Histamine has a wide distribution throughout the gastrointestinal tract, its associated organs and liver (Best, Dale, Dudley & Thorpe, 1927). It is the only substance investigated to date that has profound and qualitatively different effects on the hepatic arterial and hepatic portal vascular resistance. The molar potency and time-course of the vasodilator action of histamine on the hepatic arterial bed have been discussed previously (Richardson & Withrington, 1976b). The present experiments confirm previous observations of the effect of histamine in increasing portal vascular resistance (Greenway & Oshiro, 1973) and reveal that quantitatively it is as potent as adrenaline and noradrenaline at this site (Richardson & Withrington, 1977). The conclusion of Greenway & Oshiro (1973) that the main site of action of histamine when causing a rise in portal pressure in the cat, was by outflow block from the liver is supported by the results of work on isolated preparations of portal or mesenteric vein (Hughes & Vane, 1967; Northover, 1967; Greenway & Oshiro, 1973) and hepatic vein (Greenway & Oshiro, 1973); these reveal that the contraction of the portal vein by histamine is weak compared with that induced by noradrenaline, whilst the two substances are about equipotent on the hepatic vein. It is not possible, using the preparations described in this paper, to ascribe the effects of histamine to any particular part of the series-coupled intra- or extra-hepatic circulation, but the potency of histamine in elevating the portal venous pressure, possibly by outflow block, raises the possibility that histamine when injected intra-arterially may reach both the inflow and outflow resistance sites. This would lead not only to relaxation of the arterial resistance vessels, but also to an increase in outflow resistance. The net effect of such actions would be to decrease the pressure drop along the arterial vascular circuit, thereby reducing the fall in calculated hepatic arterial vascular resistance resulting from the intra-arterial injection of histamine.

Since histamine is destroyed by circulating histaminases it is unlikely to reach vasoactive concentrations in the systemic circulation under normal physiological conditions. However, in conditions of shock and anaphylaxis (Kahlson & Rosengren, 1971) systemic histamine levels could rise and provoke cardiovascular reactions. Hepatic arterial vasodilatation would be accompanied by vasodilatation of the gastric and intestinal (Texter, Chou, Merrill, Laureta & Frolich, 1964) and splenic beds (Davies & Withrington, 1973) leading to increased portal inflow; if histamine also induced an increase in hepatic outflow resistance this might lead to hepatic congestion and a rise in portal pressure.

The existence of two populations of histamine receptors, $H_1$ and $H_2$, in the peripheral vasculature of dogs and cats has been recognized for some time (Folkow, Haeger & Kahlson, 1948; Black *et al.*, 1972; Black *et al.*, 1975; Owen, 1975) and in the present experiments we have examined the histamine receptors that mediate the different vascular responses to histamine in the hepatic arterial and hepatic portal venous beds of the dog. In the dog, metiamide, in the absence of mepyramine, does not antagonize the depressor responses to i.v. histamine although if given after mepyramine it causes a further antagonism of these responses (Black *et al.*, 1975). A similar pattern of antagonism to the vasodilator effects of histamine has been reported in the femoral and superior mesenteric arterial vascular beds of the cat (Flynn & Owen, 1975).

In the present series of experiments, we have used the same doses of antagonists as Black *et al.* (1975) found effective in the dog, and we always observed a parallel and significant shift of the histamine dose-response curve to the right after mepyramine for both hepatic arterial vasodilation and hepatic portal vasoconstriction, without suppression of the maxima. This represents strong evidence for the existence of a population of histamine $H_1$-receptors mediating the effects in the two hepatic vascular beds. However, we found no displacement to the right of the histamine dose-response curves of the hepatic arterial or portal vascular beds during infusions of metiamide, whether these were made before or after mepyramine. In contrast, at both sites, we observed small shifts of the histamine dose-response curves to the left of the controls which might be attributable to the existence of histamine $H_2$-receptors in both vascular beds which, on stimulation, oppose the actions of $H_1$-receptor activation. The conclusion from the present experiments is that, as in the saphenous vein of the dog (Powell & Brody, 1976), in both the hepatic arterial and portal venous vascular beds of the dog, histamine $H_1$-receptors are predominantly responsible for the vascular effects of histamine, which are vasodilatation in the hepatic arterial bed, and vasoconstriction in the hepatic portal vascular bed.

In the present experiments the separate effects of bradykinin, 5-HT and histamine on the hepatic arterial and portal vascular beds have been examined. The evidence suggests that their independent physiological release would not significantly influence the total blood flow or its distribution through the liver.

However, elevated portal or systemic levels of histamine, bradykinin and 5-HT could by themselves evoke marked alterations in liver blood flow and its distribution between the arterial and portal circuits. In pathological circumstances it is unlikely that these substances are released in isolation; more probably they are released as a group together with other vasoactive substances (Lefer, 1973; Douglas, 1975). Interactions between bradykinin, 5-HT and histamine and other vasoactive materials released concomitantly (e.g. prostaglandins, glucagon, catecholamines) which are known to be highly active on the liver vasculature (Richardson & Withrington, 1976a,b,c,d; 1977) could lead to significant changes in liver perfusion.

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# SELECTIVE INHIBITORY ACTIONS OF SODIUM-*p*-BENZYL-4-[1-OXO-2-(4-CHLOROBENZYL)-3-PHENYL PROPYL] PHENYL PHOSPHONATE (N-0164) AND INDOMETHACIN ON THE BIOSYNTHESIS OF PROSTAGLANDINS AND THROMBOXANES FROM ARACHIDONIC ACID

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1 Sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl propyl]phenyl phosphonate (N-0164) selectively inhibited the formation of thromboxane-$A_2$ from prostaglandin endoperoxides by human platelet microsomes in a dose-dependent manner ($IC_{50}$ $2.2 \times 10^{-5}$ M or 11.6 µg/ml).

2 N-0164 was approximately 15 to 20 times as potent as indomethacin as an inhibitor of thromboxane-$A_2$ formation. In contrast, indomethacin was 20 times as potent as N-0164 as an inhibitor of prostaglandin endoperoxide formation from arachidonic acid ($IC_{50}$ $2.6 \times 10^{-5}$ M or 9.4 µg/ml).

3 Spiral strips of dog coronary arteries relaxed in the presence of prostaglandin endoperoxides and were contracted by prostaglandin $E_2$ and thromboxane-$A_2$ and were therefore used to distinguish between prostaglandins and their intermediate precursors, the endoperoxides.

4 Neither indomethacin nor N-0164 (both 50 µg/ml) significantly inhibited the formation of prostaglandin-like activity from the endoperoxides following incubation with indomethacin-pretreated rabbit kidney medulla microsomes.

5 It is not known whether this action of N-0164 is related to its ability to antagonize certain actions of prostaglandins (and related compounds) or whether N-0164 can penetrate the cell membrane to inhibit thromboxane formation in the intact cell.

6 Selective inhibition of thromboxane formation by drugs such as N-0164 may be useful both clinically and as a pharmacological tool to elucidate the patho-physiological roles of the thromboxanes.

## Introduction

The prostaglandin endoperoxides (prostaglandin $G_2$ and $H_2$) can be converted by thromboxane synthetase, an enzyme present in platelets, to the potent but very unstable rabbit aorta contracting substance, thromboxane $A_2$, which is known to be released during platelet aggregation (Hamberg, Svensson & Samuelsson, 1975; Bunting, Moncada, Needleman & Vane, 1976; Needleman, Moncada, Bunting, Vane, Hamberg & Samuelsson, 1976b).

Sodium *p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl propyl]phenyl phosphonate (N-0164) has been found to antagonize selectively certain actions of prostaglandins (Eakins, Rajadhyaksha & Schroer, 1976). In addition, in a preliminary series of experiments, this compound in higher concentrations was also found to inhibit the conversion of prostaglandin endoperoxides to thromboxane-$A_2$-like activity by human platelet microsomes (Kulkarni & Eakins, 1976). Only one other compound, benzydamine, has been shown to inhibit selectively thromboxane generation from prostaglandin endoperoxides at this time (Moncada, Needleman, Bunting & Vane, 1976).

In the present study, we have further investigated this action of N-0164 and compared it with indomethacin, a known prostaglandin synthetase inhibitor (Vane, 1971) on other pathways in the metabolism of arachidonic acid to prostaglandin $E_2$.

## Methods

### *Synthesis of prostaglandin endoperoxides*

Sheep seminal vesicle microsomes were prepared as described by Takeguchi, Kotina & Sih (1971) and resuspended in phosphate buffer (50 mM, pH 7.5–8). This suspension was then redistributed in 0.5 ml quantities in smaller tubes and the protein content of each sample determined by the method of Lowry, Rosebrough, Farr & Randall (1951). These microsomal preparations plus arachidonic acid were used as a prostaglandin endoperoxide generating system as described by Bunting *et al.* (1976). Seminal vesicle microsomes (2–300 μg protein) were incubated without cofactors with sodium arachidonate (2 μg) at room temperature (22°C) for 1 min, 50 μl samples of this mixture were then tested for biological activity as described below.

### *Preparation of human platelet microsomes*

Batches of expired platelet-rich human plasma were incubated with indomethacin (2 μg/ml) for 16 h then centrifuged at 2000 *g* for 15 minutes. The resultant platelets were resuspended in ice-cold 0.9% w/v NaCl solution (saline) and lysed by shell freezing and thawing (five times). Cell debris was then removed by centrifugation at 8000 *g* for 15 minutes. The supernatant was then centrifuged at 100,000 *g* for 45 minutes. The resultant microsomal pellet was resuspended in 5 ml phosphate buffer (50 mM, pH 7.5–8) and redistributed in 0.5 ml quantities and the protein content of each sample measured as before.

### *Preparation of rabbit kidney medulla microsomes*

Kidneys were excised from white albino rabbits and the kidney medulla separated from the cortex. The medulla was then cut into small pieces, homogenized in ice-cold 0.15 M KCl containing 5 μg/ml indomethacin and centrifuged at 2000 *g* for 15 minutes. The supernatant was then re-centrifuged at 100,000 *g* for 45 min and the resultant microsomal pellet resuspended in phosphate buffer (50 mM, pH 7.5–8). This suspension was then redistributed in 0.5 ml quantities in small tubes and the protein content of each sample determined as before.

### *Synthesis of thromboxane-$A_2$-like activity*

In some experiments, thromboxane-$A_2$-like activity was produced by further incubation of the prostaglandin endoperoxide suspension with the indomethacin-treated human platelet microsomes (150–250 μg protein) for 2 min at 0°C; 50 μl aliquots were then immediately tested for biological activity.

In other experiments, the authentic prostaglandin endoperoxide, prostaglandin $H_2$ was incubated with the indomethacin-treated human platelet microsomes as described above.

### *Assay of biological activity*

Bioassays were made either on a rabbit aorta (Piper & Vane, 1969) or in some experiments, on a dog coronary artery (Kulkarni, Roberts & Needleman, 1976) superfused at 10 ml/min with Krebs solution at 37°C containing a mixture of pharmacological antagonists (Gilmore, Vane & Wyllie, 1968) to block acetylcholine, 5-hydroxytryptamine, catecholamines and histamine, plus indomethacin (1 μg/ml), to inhibit prostaglandin biosynthesis by the assay tissues (Vane, 1971). Angiotensin II was used to standardize the assay tissues.

### *Drugs*

The following drugs were used: arachidonic acid, sodium salt (Sigma Chemical Company, St. Louis, Mo.); indomethacin (Merck Sharp and Dohme, Rahway, New Jersey); methysergide maleate (Sandoz, Hanover, New Jersey); diphenhydramine hydrochloride (Parke Davis); atropine sulphate (Burroughs Wellcome); propranolol (Ayerst Laboratories); phenoxybenzamine hydrochloride (Smith, Kline and French); angiotensin amide (Ciba-Geigy Laboratories); N-0164 (Nelson Research and Development Company).

Stock solutions of N-0164 up to a maximum concentration of 250 μg/ml were freshly prepared each day by dissolving the compound in warm phosphate buffer. Particular care was taken to ensure that the drug remained in solution, any signs of precipitation disappeared with further warming.

## **Results**

When sodium arachidonate was incubated without cofactors with sheep seminal vesicle microsomes at room temperature for 1 min, a product was formed which resembled the prostaglandin endoperoxides, prostaglandin $G_2$ or $H_2$. It contracted the rabbit aorta, had a half life of 2–4 min in aqueous solution at 37°C and, in addition, was converted to a more powerful rabbit aorta contracting substance when incubated at 0°C for 2 min with indomethacin-treated human platelet microsomes and immediately tested (Figure 1f, g). In other experiments, a similar activity was produced when authentic prostaglandin $H_2$ was incubated with human platelet microsomes under identical conditions (Figure 1a, b). The characteristics of this rabbit aorta contracting substance were found to be the same as those described previously (Bunting *et al.*, 1976; Needleman *et al.*, 1976b).

Addition of N-0164 (5–50 μg/ml) to the human platelet microsomes for 2 min inhibited the subsequent conversion of prostaglandin endoperoxides to thromboxane-$A_2$ in a dose-dependent manner.

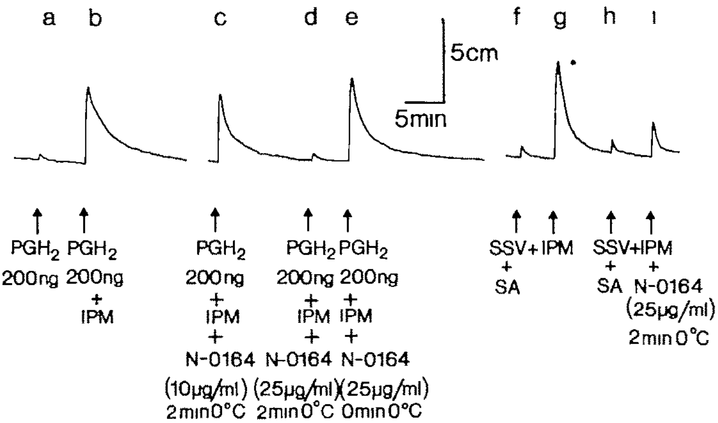

**Figure 1** Inhibition of thromboxane-$A_2$-like ($TxA_2$-like) activity by N-0164. Bioassay of rabbit aorta contracting substances (a) prostaglandin $H_2$ ($PGH_2$), 200 ng; (b) $PGH_2$ (200 ng) was incubated at 0°C for 2 min in 50 µl phosphate buffer (50 mM, pH 8) with indomethacin-treated human platelet (IPM) microsomes (200 µg protein) to yield $TxA_2$-like activity; (c and d) addition of N-0164 (10 µg/ml and 25 µg/ml) to the IPM incubate for 2 min at 0°C inhibited formation of $TxA_2$-like activity; (e) addition of N-0164 (25 µg/ml) to IPM incubate immediately before testing did not prevent the appearance of $TxA_2$-like activity, (f) sheep seminal vesicle (SSV) microsomes (250 µg protein) were incubated with 2 µg sodium arachidonate (SA) in 100 µl phosphate buffer for 1 min at 22°C and 50 µl tested; (g) further incubation of this sample at 0°C for 2 min with IPM (200 µg protein) to yield $TxA_2$-like activity; (h) same as (f); (i) addition of N-0164 (25 µg/ml) to IPM incubate for 2 min at 0°C inhibited formation of $TxA_2$-like activity

Inhibition of the formation of thromboxane-$A_2$ from authentic prostaglandin $H_2$ is seen in Figure 1c and d. It was possible that these concentrations of N-0164 could antagonize the contraction produced by thromboxane-$A_2$ on the rabbit aorta. However, this was shown not to be the case since addition of the higher concentration of N-0164 (25 µg/ml) immediately before testing (zero minute incubation) failed to inhibit the contractions induced by thromboxane-$A_2$ (Figure 1e). A similar inhibition of thromboxane-$A_2$ formation was obtained when sheep seminal vesicles and sodium arachidonate were used as the source of prostaglandin endoperoxides (Figure 1i).

In the next series of experiments, the inhibitory effects of N-0164 were compared with indomethacin on both the prostaglandin endoperoxide and the thromboxane-$A_2$ generating systems. Dose-response curves were obtained for each drug as inhibitors of prostaglandin endoperoxide formation from the sheep seminal vesicle plus sodium arachidonate incubates (Figure 2a), and as inhibitors of thromboxane-$A_2$ formation produced by the subsequent incubation with platelet microsomes (Figure 2b). In addition, thromboxane-$A_2$ formation by platelet microsomes from authentic prostaglandin $H_2$ was also examined. It can be seen that indomethacin was a potent inhibitor of endoperoxide formation, the concentration required to produce a 50% inhibition of endoperoxide formation ($IC_{50}$) being $2.6 \times 10^{-5}$ M (9.4 µg/ml); at the $IC_{20}$ level of inhibition, indomethacin was 20 times as potent as N-0164 on this preparation. However, indomethacin was much less effective than N-0164 as an inhibitor of thromboxane-$A_2$ formation. N-0164 was approximately 10 to 15 times as potent against thromboxane-$A_2$ formation ($IC_{50}$ $2.2 \times 10^{-5}$ M or 11.6 µg/ml) as it was against prostaglandin endoperoxide formation. When authentic prostaglandin $H_2$, instead of the sheep seminal vesicle incubate, was incubated with the platelet microsomes to generate thromboxane-$A_2$ activity and the inhibitory effects of N-0164 retested, the $IC_{50}$ remained virtually unchanged at 10 µg/ml.

In the final series of experiments, an attempt was made to assess the activity of N-0164 as an inhibitor of the conversion of prostaglandin endoperoxides to prostaglandin. In these experiments, bioassays were made on spiral strips of dog coronary artery which relax in the presence of prostaglandin endoperoxides generated by the sheep seminal vesicle plus arachidonate incubate and contract in the presence of thromboxane-$A_2$ and prostaglandin $E_2$ (Figure 3a, b, c). Further incubation of the endoperoxides with indomethacin-treated rabbit kidney medulla microsomes for 4 min at 22°C yielded a product, probably prostaglandin $E_2$, which contracted the coronary artery strips (Figure 3e). Equivalent quantities of boiled kidney medulla microsomes failed to produce this contractile activity. Addition of either N-0164 or indomethacin, both 50 µg/ml, to the kidney medulla microsomes for 4 min did not interfere with

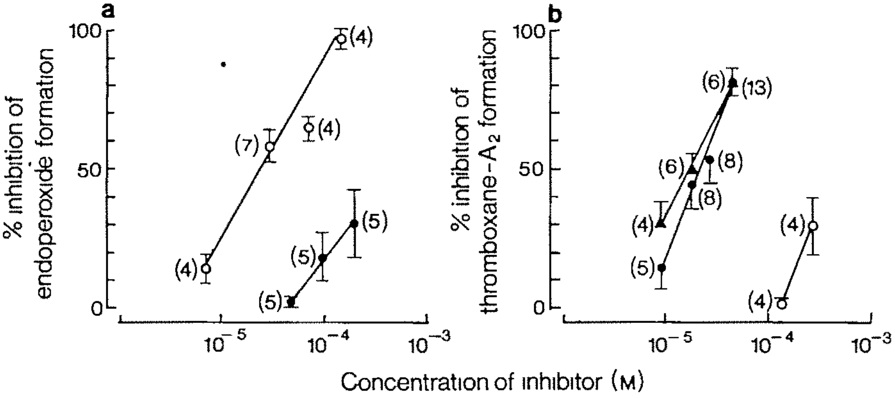

**Figure 2** A comparison of the inhibitory effects of N-0164 and indomethacin on the biosynthesis of prostaglandin endoperoxides (a) and thromboxane-$A_2$ (b). (a) Dose-response curves for indomethacin (○) and N-0164 (●) as inhibitors of prostaglandin endoperoxide formation from sheep seminal vesicle plus sodium arachidonate incubates (b) Dose-response curves for indomethacin (○) and N-0164 (●) as inhibitors of thromboxane-$A_2$ formation produced by subsequent incubation of the sheep seminal vesicles plus arachidonate with platelet microsomes. (▲) Dose-response curve for N-0164 as inhibitor of thromboxane-$A_2$ formation produced by incubation of platelet microsomes with authentic prostaglandin $H_2$ Number of observations at each point is shown in parentheses.

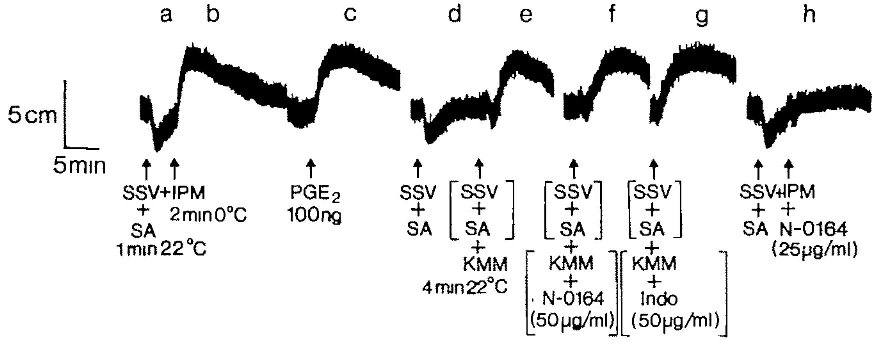

**Figure 3** N-0164 and indomethacin do not inhibit the conversion of prostaglandin endoperoxides to prostaglandin-like activity. Bioassay on dog coronary artery. (a) Sheep seminal vesicle (SSV) microsomes (246 μg protein) were incubated with 1 μg sodium arachidonate (SA) in 100 μl phosphate buffer (50 mM, pH 8) at 22°C for 1 min to generate prostaglandin endoperoxides ($PGG_2$ and $PGH_2$) which induced relaxation. (b) Further incubation with indomethacin-treated human platelet microsomes (IPM) at 0°C for 2 min generated thromboxane-$A_2$ ($TxA_2$) resulting in contraction. (c) Authentic prostaglandin $E_2$ ($PGE_2$), 100 ng, caused contraction. (d) Same as (a). (e) Same as (a) but incubated with indomethacin-treated rabbit kidney medulla microsomes (KMM) (30 μg protein) at 22°C for 4 min to generate prostaglandin-like activity. (f) Addition of N-0164 (50 μg/ml) to KMM incubate for 4 min failed to inhibit the appearance of prostaglandin-like activity (g) Addition of indomethacin (Indo, 50 μg/ml) to KMM incubate also failed to inhibit the formation of prostaglandin-like activity. (h) Addition of N-0164 (25 μg/ml) to IPM incubate for 2 min at 0°C inhibited formation of $TxA_2$-like activity.

the formation of this prostaglandin $E_2$-like substance (Figure 3f and g), although half this concentration of N-0164 (25 μg/ml) was found to inhibit considerably the formation of thromboxane-$A_2$-like activity on this bioassay tissue (Figure 3h).

## Discussion

N-0164 (0.5–5 μg/ml) has previously been found to be a potent, partially selective, antagonist of the contractile actions of both E and F prostaglandins on

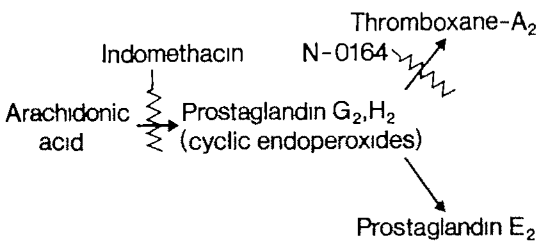

**Figure 4** Schematic representation of the major sites of actions of indomethacin and N-0164 on pathways in the biosynthesis of thromboxane-$A_2$ and prostaglandin $E_2$ from arachidonic acid.

isolated preparations of gerbil, rat and guinea-pig gastrointestinal muscle (Eakins *et al.*, 1976). However, it should be noted that N-0164 failed to antagonize selectively the relaxant action of prostaglandin $E_2$ on the rabbit isolated coeliac artery (Kulkarni & Eakins, unpublished observations). The results of the present study indicate that, in addition, higher concentrations of N-0164 (10–50 µg/ml) can selectively inhibit the formation of thromboxane-$A_2$-like activity from prostaglandin endoperoxides by human platelet microsomes.

The inhibitory action of N-0164 was studied at three possible sites of action on the pathways of arachidonic acid metabolism as outlined in Figure 4. Thus the inhibitory effect of N-0164 was determined on the conversion of arachidonic acid to prostaglandin endoperoxide by the fatty acid cyclo-oxygenase (Hamberg & Samuelsson, 1973), on the formation of thromboxane-$A_2$ from the endoperoxide by thromboxane synthetase (Hamberg *et al.*, 1975; Needleman *et al.*, 1976b) and finally on the conversion of prostaglandin endoperoxides to prostaglandin $E_2$ which involves the endoperoxide isomerase (Hamberg, Svensson & Samuelsson, 1976).

Bunting *et al.* (1976) have described the use of seminal vesicle microsomes plus arachidonate as an endoperoxide generating system coupled with platelet microsomes plus prostaglandin endoperoxides to generate thromboxane-$A_2$. The rabbit aorta contracting activity formed from the endoperoxides by the platelet microsomes in the present experiments was very labile, disappearing within 1 min in aqueous solution at 37°C. Furthermore, under control conditions, equivalent quantities of boiled platelet microsomes failed to produce the rabbit aorta contracting activity. From these characteristics, which are essentially the same as those described previously (Bunting *et al.*, 1976; Needleman *et al.*, 1976b), it was assumed that the rabbit aorta contracting activity generated in the present experiments was thromboxane-$A_2$.

Indomethacin, a known prostaglandin synthetase inhibitor (Vane, 1971), was approximately 20 times as potent as N-0164 as an inhibitor of prostaglandin endoperoxide formation from arachidonic acid. In contrast, N-0164 was approximately 10 to 15 times as potent as indomethacin in preventing the formation of thromboxane-$A_2$ from prostaglandin endoperoxides by thromboxane synthetase. The similarity between the $IC_{50}$ obtained for N-0164 as an inhibitor of thromboxane-$A_2$ generation by platelet microsomes from the authentic prostaglandin endoperoxide (10 µg/ml), compared to that derived from the sheep seminal vesicle incubate (11.6 µg/ml) provides additional confirmation of the validity of the method described by Bunting *et al.* (1976). The mechanism of this action of N-0164 is not known at present, but it may be related to the ability of the compound to block the contractile actions of prostaglandin and prostaglandin endoperoxides on certain smooth muscles (Kulkarni & Eakins, unpublished observations). It should also be noted that thromboxane synthetase is associated with the microsomal fraction in cells (Needleman *et al.*, 1976b), and it is not clear at this time whether N-0164 can penetrate and inhibit thromboxane synthetase within the intact cell.

The conversion of prostaglandin endoperoxides both to prostaglandin and thromboxane-$A_2$ was studied using the dog coronary artery as the assay tissue. Indomethacin-treated rabbit kidney medulla microsomes were used as a source of endoperoxide isomerase (Moncada, personal communication). Spiral strips of dog coronary arteries relax in the presence of prostaglandin endoperoxide but contract in response to either prostaglandin $E_2$ or thromboxane-$A_2$. Thus this preparation responds in a similar manner to human and bovine coronary arteries (Needleman, Kulkarni & Raz, 1977) and may therefore be used to distinguish between prostaglandins and their intermediate precursors, the endoperoxides. The fact that neither N-0164 nor indomethacin (both 50 µg/ml) had a significant effect on the formation of prostaglandin-like activity from the endoperoxides, although N-0164 (25 µg/ml) markedly inhibited the formation of thromboxane-$A_2$, suggests that neither drug is an effective inhibitor of the prostaglandin endoperoxide isomerase.

The importance of thromboxane-$A_2$ in pathological conditions is not well established at the present time. However, Vane (1972) has suggested that the actions of prostaglandin synthetase on arachidonic acid could lead to a whole cascade of substances, each of which may have some contribution to the inflammatory response. Furthermore, the identification of the 'rabbit aorta contracting substance' released during anaphylaxis from sensitized guinea-pig lungs (Piper & Vane, 1969) with thromboxane-$A_2$ (Hamberg *et al.*, 1975) suggests a role for these substances in anaphylactic bronchoconstriction. Thromboxanes are also released during platelet aggregation (Hamberg *et al.*, 1975; Needleman *et al.*, 1976b; Needleman, Minkes & Raz, 1976a) and may play a role in arterial vasoconstriction. Specifically it has been suggested that thromboxanes may be involved in both coronary and cerebral arterial spasm (Ellis, Oelz, Roberts, Payne, Sweetman, Nies & Oates, 1976; Needleman *et al.*, 1977).

Selective inhibition of thromboxane formation may thus be useful in treating myocardial ischemia and other conditions in which thromboxanes are involved. Finally, N-0164 may be useful as a pharmacological tool to elucidate the patho-physiological roles of the thromboxanes.

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# THE NATURE OF POTASSIUM CHLORIDE-INDUCED RELAXATIONS OF THE RAT ANOCOCCYGEUS MUSCLE

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1 The nature of KCl-induced relaxations of the rat anococcygeus muscle was investigated.

2 The relaxations were mimicked by other $K^+$ salts, but not by NaCl.

3 The muscle was more susceptible to the relaxant effects of KCl than the contractile effects.

4 Addition of ouabain (100 μM) had no effect on the relaxations.

5 The relaxations were abolished by tetrodotoxin (5 μg/ml), procaine (500 μM), and by section of the inhibitory nerves.

6 The results suggest that KCl-induced relaxations are due to stimulation of the inhibitory nerves by $K^+$.

## Introduction

The actions of potassium chloride (KCl) on the isolated anococcygeus muscle of the rat are complex (Gibson & Pollock, 1973). On the resting muscle, which is devoid of tone, KCl produces dose-related contractions, which are due partly to direct depolarization of the muscle and partly to release of endogenous noradrenaline (NA). However, when the tone of the muscle is raised by acetylcholine (ACh) or guanethidine, KCl now produces dose-dependent relaxations.

At least two possibilities might explain these rather unusual relaxations of the muscle to KCl. Firstly, since KCl can cause contraction by releasing motor transmitter (NA), it was possible that the relaxations might be due to release of inhibitory transmitter thought to exist in this tissue (Gillespie, 1972). Secondly, it has been proposed that KCl might inhibit muscle activity by stimulating Na/K-adenosine triphosphatase (ATPase) activity in the cell membrane, thus causing hyperpolarization (Shibata, Fukuda, & Kurahashi, 1973; Johns & Paton, 1974).

The object of the present study was to characterize the nature of these KCl-induced relaxations of the anococcygeus muscle and to determine which, if either, of the above mechanisms might be involved.

A preliminary account of part of this work was given to the British Pharmacological Society (Gibson & James, 1976).

## Methods

Male Wistar rats (200–300 g) were killed by stunning and exsanguination. The two anococcygeus muscles were then suspended in a 30 ml organ bath containing Krebs bicarbonate solution (mM: NaCl 118.1; KCl 4.7; $MgSO_4$ 1.0; $KH_2PO_4$ 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25.0; and glucose 11.1) which was maintained at 37°C and gassed continuously with 95% $O_2$ and 5% $CO_2$. A resting tension of 0.5 g was placed on the muscle and changes in tension were detected by a Washington isometric transducer and recorded by a Washington 400 MD/2 pen recorder.

To evoke field stimulation the muscles were threaded through a pair of platinum electrodes embedded in Araldite. These were connected to a Palmer square wave pulse generator, which was used to stimulate the muscle at a frequency of 10 Hz, 1 ms, for 15 s, using a supramaximal voltage.

In order to produce relaxations to KCl, muscle tone was first raised by addition of ACh (40 μM). In most cases phentolamine (1 μM) was also added to offset any effect due to release of NA.

### *Denervation studies*

Animals were anaesthetized with pentobarbitone (40 mg/kg). The anococcygeus muscles were approached via the scrotum, as outlined by Gillespie & McGrath (1973).

The object of these studies was to cut, if possible, the inhibitory fibres running to the anococcygeus muscle. However, since the exact neural pathways to the muscle have not yet been fully elucidated we could not be certain of the selectivity of our technique. Nevertheless it is thought that the inhibitory pathway enters the muscle on its ventral aspect, near the region where the two muscles unite. Nerve-like structures

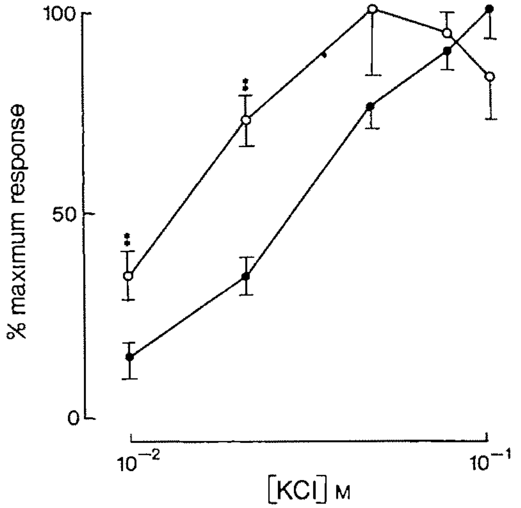

**Figure 1** Dose-response curve of the contractile (●) and relaxant (O) effects of KCl on the rat anococcygeus muscle. Each point is the mean of at least six observations; vertical bars show s.e. mean. $^{**}=0.01 > P > 0.001$.

were sectioned in this area, and in addition the connective tissue between the colon and the anococcygeus was split. A further nerve pathway was seen to enter the muscle towards the spinal tendon. This nerve ran in close apposition to an artery and was left untouched.

One muscle in each animal was treated as above; the other was left untouched and served as a control.

Operated animals were then allowed 3 days to recover. On the 4th day the rats were killed, and the anococcygeus muscles dissected.

### *Drugs*

The following drugs were used: acetylcholine chloride (Koch-Light), ouabain (Sigma), pentobarbitone sodium (Abbott), phentolamine mesylate (Ciba), procaine hydrochloride (Evans Medical) and tetrodotoxin (Sigma).

## Results

### *Characteristics of relaxations*

In order to compare the relative effectiveness of KCl in producing either contraction or relaxation of the anococcygeus muscle dose-response curves were obtained for each effect (Figure 1). These suggested that the muscle was more susceptible to the relaxant actions of KCl.

The relaxations produced by KCl were mimicked by other $K^+$ salts, including bicarbonate and tartrate (Figure 2). However, NaCl did not cause relaxation, but if anything produced a further increase of muscle tone.

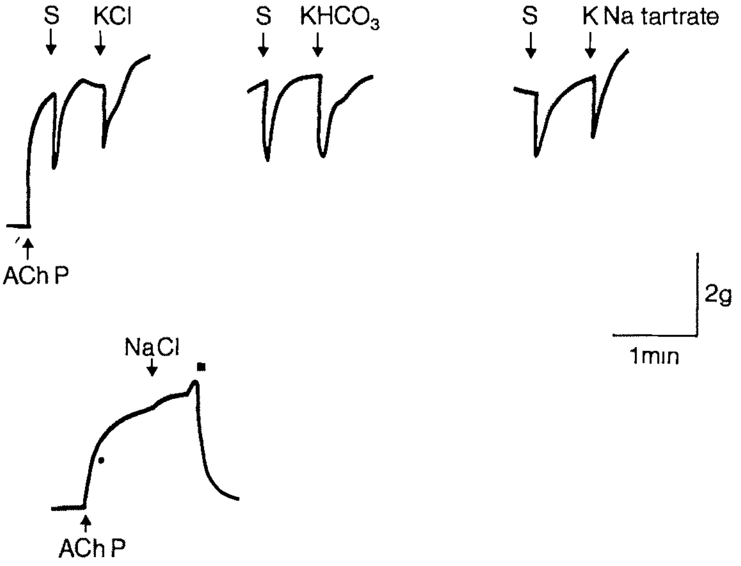

**Figure 2** Effect of different salt solutions on the contracted anococcygeus muscle. S represents field stimulation at 10 Hz. At AChP, muscle tone was raised by acetylcholine (40 µM), in the presence of phentolamine (1 µM). All salts were added at a concentration of 20 mM.

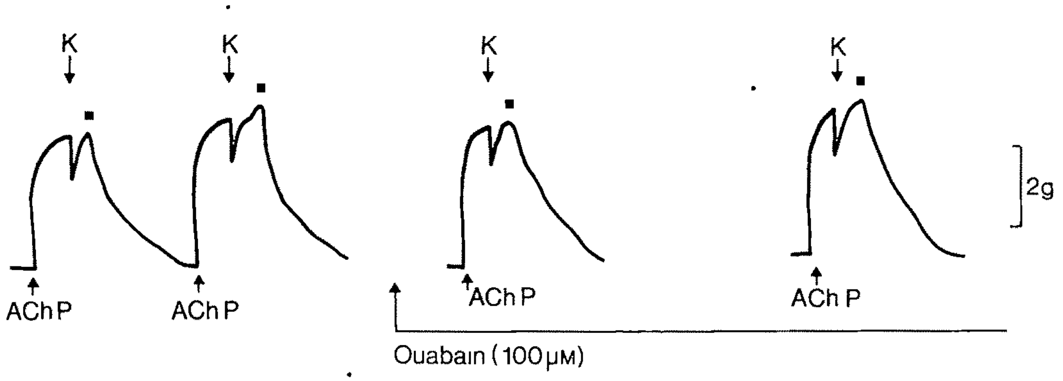

**Figure 3** Effect of ouabain on KCl-induced relaxations of the anococcygeus muscle At AChP muscle tone was raised by acetylcholine (40 μM) in the presence of phentolamine (1 μM) At K, KCl (20 mM) was added: ■ represents washout. The third and fourth traces were obtained 15 min and 30 min respectively after addition of ouabain (100 μM).

*Effect of ouabain*

Addition of ouabain (100 μM) to the bathing medium did not affect the relaxations produced by KCl, even after an incubation period of 30 min (Figure 3).

*Effect of tetrodotoxin*

Figure 4a shows the effect of tetrodotoxin (TTX) on KCl-induced relaxations of the muscle. TTX (5 μg/ml) produced a complete block of the relaxations, which returned when the TTX was washed out of the bath.

*Effect of procaine*

Before administration of procaine, the muscle was responding with relaxations to nerve stimulation and KCl (Figure 4b). However, procaine (500 μM) blocked

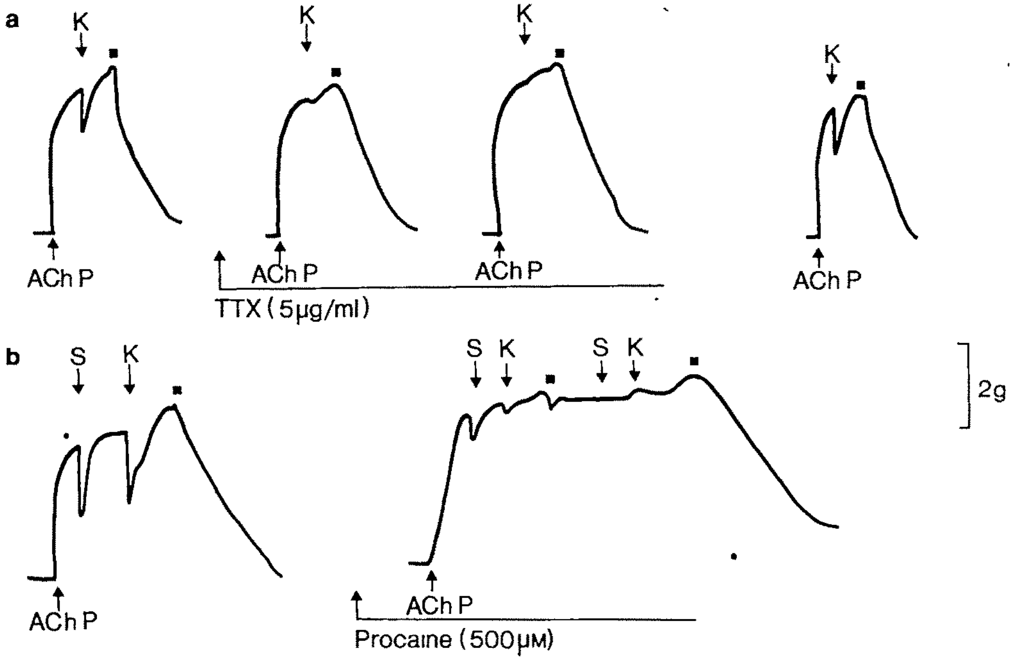

**Figure 4** Effect of (a) tetrodotoxin and (b) procaine on KCl-induced relaxations. At AChP tone was raised with acetylcholine (40 μM) in the presence of phentolamine (1 μM). K represents addition of KCl (20 mM), S field stimulation (10 Hz, 1 ms, 15 s), and ■ washout.

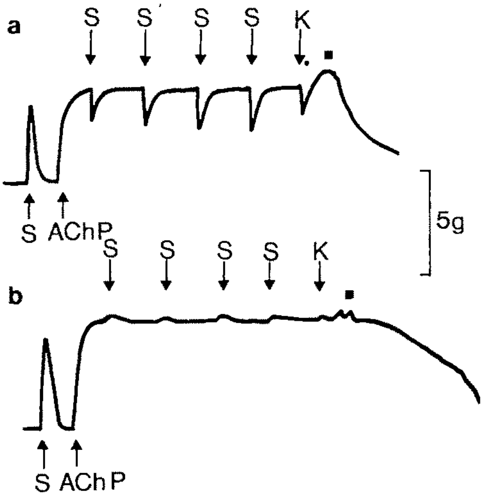

**Figure 5** Effect of denervation on KCl-induced relaxations: (a) is from a control muscle, (b) from the contralateral denervated muscle. S represents field stimulation (10 Hz, 1 ms, 15s); AChP, addition of acetylcholine (40 μM) and phentolamine (1 μM); K addition of KCl (20 mM) and ■ washout.

the response to both procedures, although it had no effect on the background tone of the muscle nor on the relaxation following washout of ACh.

### *Effect of denervation*

The contralateral control muscle responded to field stimulation with contraction (Figure 5a). When muscle tone was raised by ACh, field stimulation produced relaxations which were mimicked by KCl.

The 'denervated' muscle also responded to field stimulation with contraction (Figure 4b). This contraction could be abolished by phentolamine. In addition it was found that guanethidine (30 μM) retained its indirect sympathomimetic activity on the muscle. However, when muscle tone was raised by ACh, neither field stimulation nor KCl caused relaxations (Figure 4b).

## Discussion

The main purpose of this study was to determine whether stimulation of Na/K ATPase or release of inhibitory transmitter might explain KCl-induced relaxations of the contracted anococcygeus muscle. The former possibility was tested using the Na/K ATPase inhibitor ouabain, which has been shown to prevent KCl-induced inhibitions of guinea-pig taenia coli (Shibata *et al.*, 1973) and of rabbit myometrium (Johns & Paton, 1974). However, in the rat anococcygeus muscle ouabain had no effect on the relaxations produced by KCl, although the concentration was 100 times that used in the previous studies. It would seem therefore that stimulation of Na/K ATPase activity is unlikely to explain the actions of KCl on the anococcygeus muscle.

The possibility that the release of an inhibitory transmitter explains KCl-induced relaxations was investigated in several ways. Firstly, tetrodotoxin, which has been shown to block the inhibitory response to field stimulation (Gillespie, 1972), completely blocked the KCl-induced relaxations. Secondly, the local anaesthetic procaine blocked both the response to field stimulation and to KCl. These results suggest that the relaxations to KCl are mediated through stimulation of a neural pathway, and therefore are probably due to release of the inhibitory transmitter.

However, to substantiate this probability further, the denervation studies were attempted. As stated previously, the exact details of the innervation pattern of the anococcygeus muscle is uncertain, and there is no histological method for identifying the inhibitory nerve terminals. Consequently, the only measure of a successful denervation was lack of relaxation to field stimulation, which was achieved in six out of seven experiments. In denervated muscles, addition of KCl to the bathing medium did not produce relaxations, supporting the theory that this effect of KCl is due to release of the inhibitory transmitter.

It is of interest that it is possible to destroy selectively the inhibitory fibres to the anococcygeus muscle, suggesting that there are two distinct pathways. The presence of the sympathetic nerves in denervated muscles was confirmed by contraction to nerve stimulation, and to the indirect sympathomimetic actions of guanethidine, both effects being antagonized by phentolamine. Thus, two of the criteria for the presence of adrenergic fibres in the anococcygeus muscle were satisfied (Gibson & Gillespie, 1973). The third criterion, demonstration of the presence of adrenergic fibres by fluorescence microscopy, was not attempted in this study.

It is possible that the sympathetic fibres are contained in the nerve seen to enter the muscle in close proximity to an artery, since this nerve was left untouched. Alternatively, McKirdy & Muir (1976) have suggested the presence of a ganglion in the motor pathway adjacent to the muscle, and thus it is possible that in the present experiments the sympathetic tract was cut preganglionically. In the case of the inhibitory nerves, it seems that the ganglion in this pathway (Gilliespie & McGrath, 1973) does not lie in close apposition to the muscle, since nerve section abolished responses to field stimulation.

Although chloride ions have been shown to be involved in both hyperpolarization and depolarization (Marshall, 1973; Constanti & Nistri, 1976) the active ion in KCl producing relaxation of the anococcygeus muscle is $K^+$, since the effect was mimicked by other

$K^+$ salts, but not by NaCl. Since the dose-response for the relaxant effects of KCl was to the left of that for the contractile effects, it would seem that the inhibitory nerves are more susceptible to depolarization by KCl than the motor nerves. In the case of field stimulation, the inhibitory nerves function at a lower frequency than the motor nerves (Gillespie, 1972; Gibson & Gillespie, 1973).

In conclusion, KCl-induced relaxations of the rat anococcygeus muscle appear to be due to stimulation of the inhibitory nerves by $K^+$, causing release of an unknown inhibitory transmitter.

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# THE EFFECTS OF GASTRIN AND GASTRIN ANALOGUES ON PANCREATIC ACINAR CELL MEMBRANE POTENTIAL AND RESISTANCE

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1 Intracellular recordings of membrane potentials and input resistance have been made from the exocrine acinar cells of mouse and rat pancreas placed in a tissue bath perfused with Krebs-Henseleit solution.

2 The resting acinar cell membrane potential was about −38 mV. The acinar cells were stimulated by cholecystokinin-pancreozymin (CCK-PZ), gastrin and the gastrin-related polypeptides, caerulein and desulphated caerulein. The immediate effect of stimulation with these secretagogues was always a depolarization and a concomitant reduction in input resistance and time constant. Depolarization of the acinar cell membrane by these secretagogues was not abolished in the presence of atropine (1.4 μM).

3 These peptide secretagogues were divided into the gastrin group and the CCK-PZ group according to the time course of the depolarizations and the shape of the dose-response curve. The depolarization evoked by the gastrin group returned quickly to the resting level but that evoked by the CCK-PZ group was long lasting. The time course and the dose-response curve for desulphated caerulein was identical with that of gastrin.

4 It was confirmed electrophysiologically that the activity of gastrin is exerted by the C-terminal tetrapeptide; but the activity of caerulein depends on the C-terminal heptapeptide, especially the presence in the molecule of the sulphated tyrosyl residue at position 7 (numbering from the C-terminus). The equivalent sulphated tyrosyl residue in CCK-PZ is probably necessary for optimal activity of this polypeptide.

5 The dose-response curves obtained by electrophysiological methods indicated that the relative potencies of the peptides on mouse pancreatic acinar cells were caerulein > CCK-PZ > gastrin. Synthetic human gastrin I was found to have a higher potency than either tetra- or pentagastrin.

## Introduction

Protein secretion from pancreatic acinar cells is stimulated by the neurotransmitter acetylcholine (ACh) (vagal nerve) and the intestinal hormone cholecystokinin-pancreozymin (CCK-PZ) (Harper, 1967). Dean & Matthews (1972) showed that electrical stimulation of the pancreatic nerve, and CCK-PZ, depolarized the pancreatic acinar cell membrane. ACh and CCK-PZ act on the acinar cell membrane by increasing the membrane conductance (Petersen, 1976). The ACh null (reversal) potential is about −10 mV (Iwatsuki & Petersen, 1976) and since the ACh-evoked depolarization is sensitive to change in extracellular Na and K concentration the mechanism underlying the ACh effect is probably an increase in both Na and K permeability (Nishiyama & Petersen, 1975). It appears that ACh, CCK-PZ and gastrin act in qualitatively the same way (Petersen & Ueda, 1975).

Gastrin and caerulein (Cn) are peptides with similar structure to the C-terminal pentapeptide of CCK-PZ. CCK-PZ and Cn, having a sulphated tyrosine residue at position 7 from the C-terminus, have a similar spectrum of biological activity (Anastasi, Erspamer & Endean, 1968; Erspamer, 1970). It is now clear that these chemical structures have a close relationship with the biological activity. A study by Tracy & Gregory (1964) of the physiological properties of synthetic peptide derivatives corresponding to various portions of the molecule of gastrin I led to the discovery that of the 17 amino acid residues present, only the C-terminal tetrapeptide amide, try-met-asp-phe-$NH_2$, was required for display of all the actions of the total molecule. They also showed that this tetrapeptide had a stimulatory effect on amylase secretion by the pancreas. On the other hand, Cn displays a potent stimulatory action on pancreatic secretion similar to that of CCK-PZ. It was reported that this potent activity was caused by the sulphated tyrosine residue at position 7 from the C-terminus (Bertaccini & Erspamer, 1969).

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The present paper concerns the relationship between the biological activity and the primary structure of Cn and gastrin as revealed by electrophysiological studies on mouse and rat pancreas segments superfused *in vitro*.

## Methods

The experiments were performed on isolated segments of pancreas from mice and, in a few cases, rats. The segments were placed in a perspex tissue bath (volume 20 ml) which was perfused with a Krebs-Henseleit solution warmed to 37°C and flowing at a rate of about 10 ml/min (Dean & Matthews, 1972; Nishiyama & Petersen, 1974). The solution had the following composition (mM): NaCl 103, KCl 4.7, $CaCl_2$ 2.56, $MgCl_2$ 1.13, $NaHCO_3$ 25, $NaH_2PO_4$ 1.15, D-glucose 2.8, Na pyruvate 4.9, Na fumarate 2.7 and Na glutamate 2.7; it was gassed with 95% $O_2$, and 5% $CO_2$. Glass microelectrodes filled with 3 M KCl + 10 mM K-citrate, having a tip resistance of about 20 to 30 MΩ, were used for the measurement of cellular transmembrane potential. Measurements of membrane potential and resistance were made by methods already described in detail by Nishiyama & Petersen (1974). One microelectrode was used for both current injection and recording. Electrode tip resistance compensation was carried out before cell impalement by adjusting the balance output of the electrometer amplifier. The proper balance of the bridge could be verified by looking at the shape of the current pulse induced change in membrane potential.

Stimulation of the tissue was done in the following way. Drugs were added directly to the bath as a single drop (0.01 ml) of a concentrated solution close to the tissue with a 1 ml syringe connected to a small needle (27¾ gauge, diameter 0.4 mm). The doses given are the amounts of drug contained in the single drops.

Drugs were obtained from the following sources: CCK-PZ (mol. wt. 3883) from Boots; 1 mg is equivalent to 3000 Crick-Harper Raper Units; synthetic human gastrin I (mol. wt. 2089) from ICI, BOC pentapeptide (mol. wt. 767.9) from Calbiochem, U.S.A., AOC tetrapeptide (mol. wt. 730.55) from Nihonkayaku Ltd (Tokyo, Japan), caerulein (mol. wt. 1352) and desulphated caerulein (mol. wt. 1272) from Farmitalia, Italy, through Kyowa Hakko Kogyo Ltd, Japan. The amino acid sequences of these peptides are shown in Table 1.

## Results

### *The resting membrane potential*

In the mouse pancreas the mean membrane potential was $-37.5 \pm 0.9$ ($n = 370$) and in the rat pancreas the comparable values were $-37.5 \pm 0.9$ ($n = 50$). These values were very similar to results previously described by Dean & Matthews (1972), Matthews & Petersen (1973) and Nishiyama & Petersen (1974) from the same preparation.

### *The effects of acetylcholine and cholecystokinin-pancreozymin*

Previous reports (Matthews & Petersen, 1973; Matthews, Petersen & Williams, 1973) showed that the dose-response curves calculated from continuous exposure of mouse pancreatic acinar cells to either ACh or CCK-PZ had shapes very similar to each other.

We used routinely a single dose of drugs added directly into the tissue bath close to the tissue and the initial experiments were designed to allow comparison between responses obtained using this technique and those previously published.

*The effects of acetylcholine* The magnitude of maximum depolarization and the time course were approximately similar to those reported by Matthews & Petersen (1973) but the dose-response curve was different in that it had a lesser slope and the initial response was seen at a dose of only $10^{-14}$ mol. The different experimental results can possibly be

**Table 1** Structure of the peptides studied

| *Peptide* | *Structure* |
|---|---|
| Human gastrin I | Pyr·Gly·Pro·Trp·Leu·Glu·Glu·Glu·Glu·Glu·Ala·Tyr·Gly·Trp Met·Asp·Phe·$NH_2$ |
| AOC tetrapeptide | t-Amyloxycarbonyl·Trp·Met·Asp·Phe·$NH_2$ |
| BOC pentapeptide | t-Butyloxycarbonyl·$\beta$·Ala·Trp·Met·Asp·Phe·$NH_2$ |
| Caerulein | Pyr·Glu·Asp·Tyr·Thr·Gly·Trp·Met·Asp·Phe·$NH_2$<br>Ȯ·$SO_3H$ |
| Desulphated caerulein | Pyr·Glu·Asp·Tyr·Thr·Trp·Met·Asp·Phe·$NH_2$ |
| CCK-PZ | Lys·Ala·Pro·Ser·Gly·Arg·Val·Ser·Met·Ile·Lys·Asp·Leu·Ser·Leu·Asp·<br>Pro·Ser·His·Arg Ile·Ser·Asp·Arg·Asp Tyr·Met·Gly·Trp·Met·Asp·Phe·$NH_6$<br>Ȯ·$SO_3H$ |

Gastrin II is identical to gastrin I, but the tyrosin residue is sulphated.

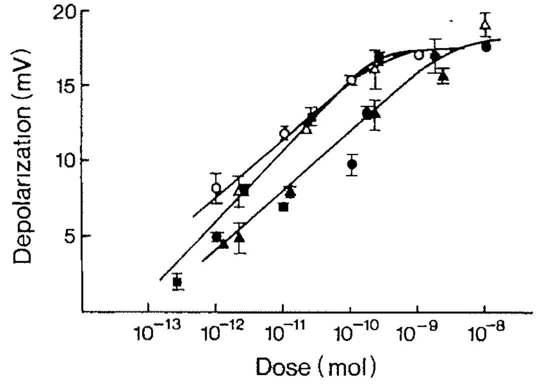

**Figure 1** Dose-response curves for the depolarization effect of gastrin analogues on the pancreatic acinar cells of mouse and rat. Mean values ($n=4-13$) are given. Vertical bars show s.e. means. (■) Synthetic human gastrin I, (●) gastrin pentapeptide, mouse; (O) gastrin pentapeptide, rat; (▲) gastrin tetrapeptide, mouse, (△) gastrin tetrapeptide, rat. In this and the following figures, 'Dose (mol)' refers to the amount of agent added to the tissue bath (capacity 20 ml) in a volume of 0.01 ml

explained by the concentration change caused by dilution at the tissue surface. In all experiments, the effect of ACh was to cause depolarization associated with a marked reduction of input resistance and time constant.

*The effects of cholecystokinin-pancreozymin* The dose-response curve was very similar to that of Matthews & Petersen (1973). The initial depolarization was seen at a dose of $10^{-15}$ mol and the magnitude of maximum depolarization was 15 mV at a dose of $10^{-11}$ mol. The depolarization was long lasting with doses of CCK-PZ over $10^{-12}$ mol. Signs of electrical uncoupling (Petersen, 1976) were sometimes seen. During the stage of uncoupling it was difficult to know the exact magnitude of depolarization. In a previous report (Nishiyama & Petersen, 1974) it was shown that CCK-PZ stimulation causes depolarization accompanied by a reduction in input resistance and time constant. We confirmed these results in all cases.

### *The effects of gastrin*

Figure 1 shows the dose-response curves for the depolarizing effect of three forms of gastrin. The curve for tetragastrin was indistinguishable from that for pentagastrin. The threshold dose of tetra- and pentagastrin was $10^{-12}$ mol and maximal depolarization was produced by a dose of $10^{-9}$ mol of these peptides. The maximum value of depolarization evoked by tetra- and pentagastrin was 18 mV. Depolarization was accompanied by a marked reduction in input resistance (Figure 2). On the other hand the synthetic human gastrin I (SHG I) had a slightly higher potency because an initial depolarization was seen at the dose of $10^{-13}$ mol and maximal effect was produced by $10^{-10}$ mol SHG I. The effect of tetra- and pentagastrin was also tested using rat pancreas. The response pattern was the same as in the mice but the dose-response curve was shifted slightly to the left showing that rat pancreatic acinar cells are more sensitive to gastrin than are those of mice.

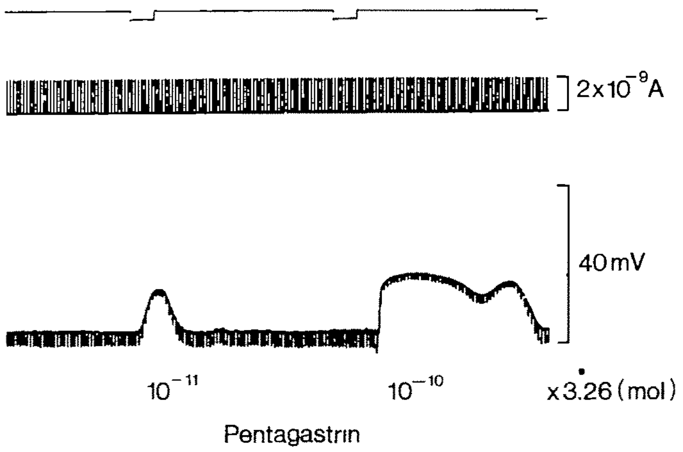

**Figure 2** Effect of pentagastrin, added to the tissue bath, on membrane potential and input resistance of rat pancreatic acinar cells The upper trace shows the time marker, the interval between signals was 1 minute. Rectangular current pulses of 100 ms duration were injected through the recording micro-electrode as indicated by the middle trace.

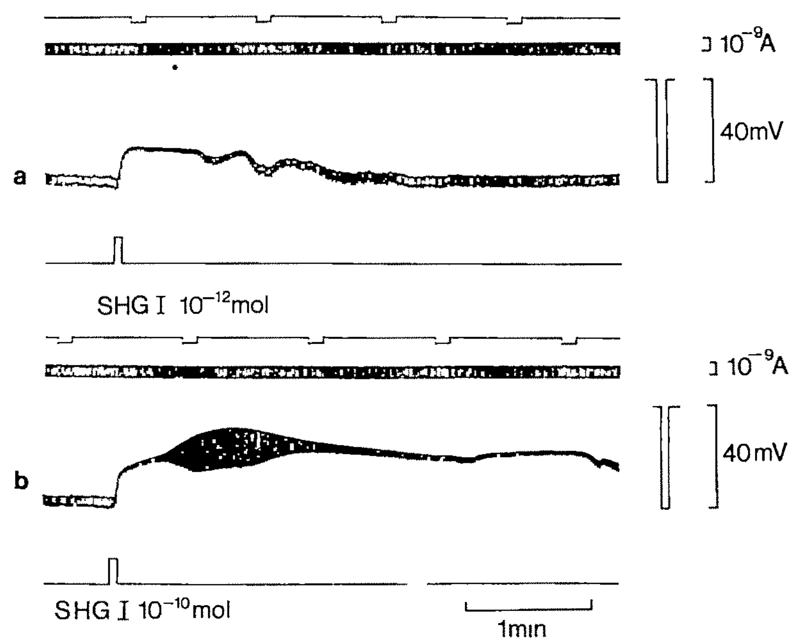

**Figure 3** The effect of synthetic human gastrin I (SHGI) on membrane potential and input resistance of rat pancreatic acinar cells. The upper record (a) shows the result of the addition of a small dose of SHGI to the tissue bath and the lower record (b) shows the effects of a larger dose of SHGI In each record the top trace is time marker (1 min intervals), the second trace indicates the injection of rectangular current pulses (100 ms duration) through the micro-electrode, the third trace is the potential recording and the lowest trace is an event marker showing the addition of drug to the bath.

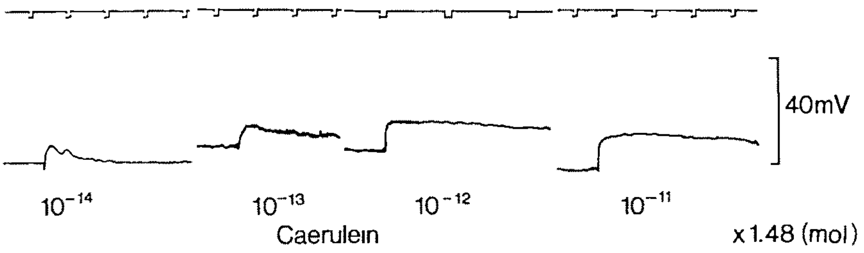

**Figure 4** Dose-dependent depolarization of mouse pancreatic acinar cells produced by caerulein. The upper trace shows the time marker (interval between signals is 1 minute).

The decrease in resistance accompanying the initial depolarization was associated with a reduction of the membrane time constant in both mouse and rat pancreatic acinar cells. In some cases the initial depolarization was followed by a period of marked increase in input resistance (Figure 3b). The time constant during the delayed depolarization was shorter than in the resting state in spite of the increased resistance. Petersen & Ueda (1976) and Iwatsuki & Petersen (1976) have shown that pancreatic acinar cells are electrically coupled. The phenomenon observed here of an increase in resistance without an accompanying increase in time constant is indicative of acinar cell uncoupling during the gastrin-induced depolarization. Depolarization of the acinar cell membrane evoked by gastrin was not abolished in the presence of atropine (1.4 μM).

### *The effect of caerulein and desulphated caerulein*

Caerulein (Cn) produced a dose-dependent depolarization of the acinar cells (Figure 4). The initial

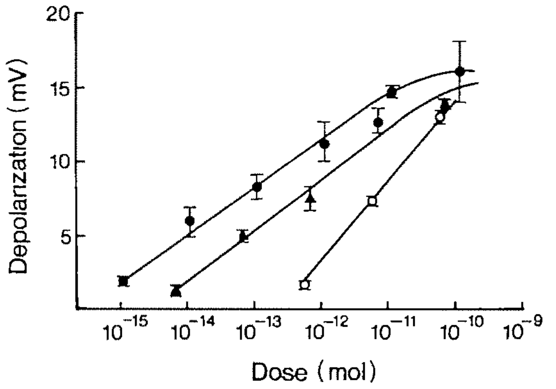

**Figure 5** Dose-response curves for the depolarizing effect of cholecystokinin-pancreozymin (▲), caerulein (●) and desulphated caerulein (○) on mouse pancreatic acinar cells. Each point is the mean of between four and fifteen measurements; vertical bars show s.e. means.

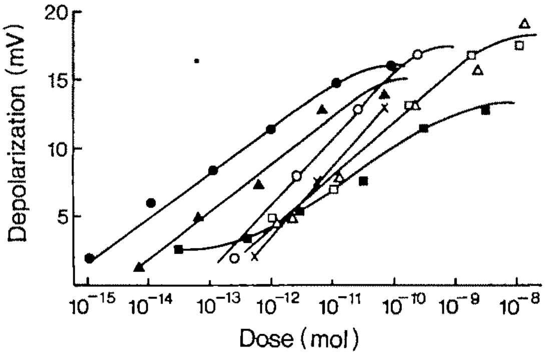

**Figure 7** Dose-response curves for depolarizing effects of secretagogues studied on mouse pancreatic acinar cells: (■) ACh; (▲) cholecystokinin-pancreozymin; (●) caerulein; (×) desulphated caerulein; (□) gastrin tetrapeptide; (△) gastrin pentapeptide; (○) synthetic human gastrin I. Each point shows mean value ($n = 4-18$).

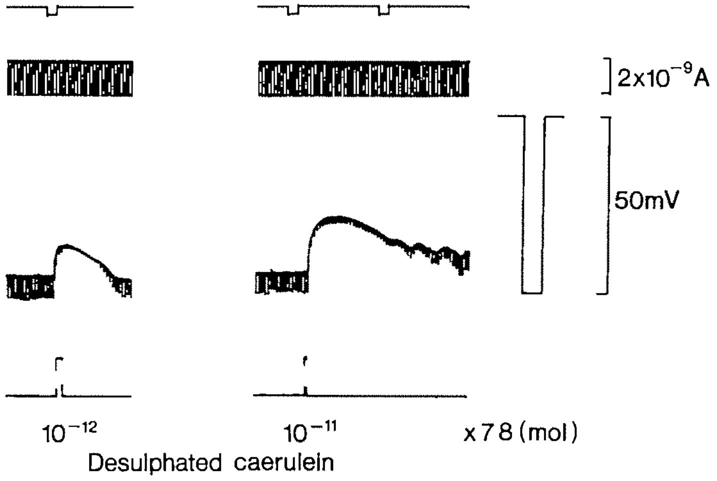

**Figure 6** The effect of desulphated caerulein on the membrane potential and resistance measurement of mouse pancreatic acinar cells. The traces are arranged as in Figure 3.

depolarization appeared at a dose of $10^{-15}$ mol and the maximum depolarization was $16 \pm 0.2$ mV at a dose of $10^{-10}$ mol. Caerulein caused depolarization of both mouse and rat pancreatic acinar cell mebrane and the dose-response curve had a shape more like that for CCK-PZ than that for ACh (Figures 5 and 7). Doses of Cn in excess of $10^{-12}$ were followed by long-lasting depolarization which had not returned to the resting potential 1 h later. Depolarization caused by $10^{-12}$ mol Cn returned to the level of the resting potential after about 20 minutes. In many cases the initial depolarization was followed by a further depolarization which, however, was accompanied by marked increase in input resistance without a corresponding increase in time constant, indicating electrical uncoupling of acinar cells. The presence of atropine (1.4 μM) in the Krebs-Henseleit solution reduced the uncoupling produced by Cn. Ueda (1974) has reported that intravenous administration of atropine reduces the Cn-induced amylase secretion by the pancreas *in vivo*. The relationship between the effect of atropine in decreasing both electrical uncoupling and amylase secretion induced by Cn is not clear.

Desulphated caerulein (d-Cn) which lacks a sulphate group on the tyrosine residue at position 7 evoked a dose-dependent depolarization of the pancreatic acinar cell membrane. The dose-response curve (Figure 5) had a shape rather similar to that for gastrin. Depolarization was always associated with a marked reduction in input resistance. Figure 6 shows the effect of d-Cn on membrane potential and resistance. The response is surprisingly similar to that induced by gastrin. This finding suggests that sulphated tyrosine residue at the position 7 is necessary for the activity characteristic of Cn and that desulphated caerulein acts as gastrin tetrapeptide. Depolarizations of the acinar cell membrane evoked by Cn and d-Cn were not abolished in the presence of atropine. Figure 5 summarizes the dose-response curves of Cn, d-Cn and CCK-PZ.

## Discussion

The essential finding in this study is that two stimulants of pancreatic protein secretion, caerulein and desulphated caerulein, reduced considerably the acinar cell membrane potential and input resistance in both mouse and rat, and that the effects of caerulein were similar to the effects of CCK-PZ. On the other hand the effects of desulphated caerulein were very similar to the effects of gastrin on acinar cell potential and resistance. We may therefore conclude that these agonists also act on acinar cells by increasing the permeability to Na and K.

In 1964 Tracy & Gregory described the isolation from hog antral mucosa of two very similar polypeptide hormones (gastrin I and II), both of which evoked a remarkable range of physiological responses from alimentary tract structures. Anderson, Barton, Gregory, Hardy, Kenner, Macleod, Preston & Sheppard (1964) succeeded in synthesizing gastrin in the same year. Tracy & Gregory (1964) studied the actions of gastrin I and II and discovered that they stimulated both exocrine pancreatic and gastric acid secretion in dogs. They discovered that of the 17 amino acid residues present, only the C-terminal tetrapeptide amide, try-met-asp-phe-$NH_2$, was required for the display of all the actions of the total molecule and that the activity of the tetrapeptide was almost entirely abolished by removal of the C-terminal amide group. Lengthening the peptide chain in the direction of the N-terminus increased the potency but did not alter the general pattern of biological activity. The extended study by Morley, Tracy & Gregory (1965), of the properties of a large series of analogues of the C-terminal tetra- and pentapeptide amides showed that the most important feature of the molecules was the aspartic carboxylic terminal amide group. In the anaesthetized cat pentagastrin is less potent than gastrin II in evoking pancreatic amylase secretion (Beswick, 1968). From the results in Figure 1 it can be seen that gastrin I has a higher potency with regard to acinar cell membrane depolarization than either tetragastrin or pentagastrin.

The polypeptide hormones gastrin I and II have the same effect on protein and fluid secretion from the rat pancreas as CCK-PZ (Dockray, 1973). Electrophysiologically the time course of depolarization evoked by gastrin (gastrin I and tetra- and pentagastrin) was very similar to that of ACh and the effect of gastrin was fully reversible in a relatively short time. However the depolarization evoked by CCK-PZ is sustained for more than 30 minutes. The effect of gastrin, as well as of CCK-PZ, was not blocked by atropine in a concentration sufficient to abolish ACh-induced depolarization. A previous report (Matthews & Petersen, 1973) and our results suggest the existence of two distinct membrane receptors for these agonists. It is indicated, however, that the affinity for the membrane receptor of CCK-PZ is greater than that of gastrin.

The decapeptide caerulein, obtained for the first time in a pure form from methanol extracts of the skin of *Hyla caerulea* displays a potent, and relatively long lasting, hypotensive action in the dog, potently stimulates some extravascular muscles and possesses a remarkable action on several external secretions of the digestive tract including exocrine pancreatic secretion in the dog (Erspamer, Roseghini, Endean & Anastasi, 1966). The juice produced by caerulein is rich in enzymes, like that produced by CCK-PZ, and 1 µg Cn in equiactive to 30 to 40 µg human gastrin I, and 10 to 20 µg pure CCK-PZ (Erspamer, Bertaccini, De Caro, Endean & Impicciatore, 1967).

The amino acid sequences described in Table 1 show the striking resemblance in structure existing between caerulein, CCK-PZ and the C-terminal pentapeptide of gastrin I. Initial depolarization induced by caerulein is seen following relatively smaller doses than that of CCK-PZ but the magnitude of the maximum depolarization and the time course of the effects produced by these two peptides is very similar. On a molar basis, caerulein is 10 to 30 times as active as gastrin I and 3 to 6 times as active as CCK-PZ on pancreatic volume flow and amylase output in the dog (Bertaccini *et al.*, 1969; Stening & Grossman, 1969). In the present study (Figure 7) caerulein is about 7 times as potent as CCK-PZ and synthetic human gastrin I about one-tenth as potent as CCK-PZ. Accurate relative potencies between CCK-PZ and gastrin I could not be estimated because the two dose-response curves were not parallel. Adding the N-terminus of gastrin to tetrapeptide amide increased its potency. In contrast, the addition of amino acids to the N-terminus of the octapeptide caerulein to form CCK-PZ resulted in a decrease in potency.

Whereas the activity of gastrin depends largely on the C-terminal tetrapeptides, the activity of caerulein and CCK-PZ depends on the C-terminal heptapeptide, an especially necessary prerequisite for activity being the presence of O-sulphated tyrosyl residue at position 7 from the C-terminus. A shift of this residue towards the C-terminus, as in gastrin, produced a reduction in biological activity. Desulphated caerulein was about 20 times less active on the pancreatic secretion and 6 to 20 times less active on gastric secretion, and 160 times less active on gallbladder contraction in the dog (Johnson, Stening & Grossman, 1969; Anastasi, Bernardi, Bertaccini, Bosisio, De Castiglione, Erspamer, Gaffredo & Impicciatore, 1968). The drastic decrease in potency due to desulphation of caerulein is seen in Figure 5. The initial depolarization evoked by caerulein occurs at a dose of $10^{-15}$ mol and that evoked by desulphated caerulein occurs at a dose of more than $10^{-13}$ mol. The dose-response curve of desulphated caerulein has a shape rather similar to that of gastrin (Figure 7). Furthermore, the time course of depolarization evoked by desulphated caerulein was surprisingly similar to that of gastrin (Figure 6), never long-lasting even if the dose was increased. The frequency of electrical uncoupling by these two peptides is nearly the same. It was concluded from the above electrophysiological results that the biological activity of desulphated caerulein was dependent on only the C-terminal tetrapeptide amide, and desulphated caerulein acted as gastrin tetrapeptide on pancreatic acinar cells. It was confirmed that the presence of a sulphated tyrosyl residue at position 7 from the C-terminus is a necessary prerequisite for the manifestation of the CCK-PZ-like actions of caerulein and the equivalent residue in CCK-PZ is possibly also required for the activity of these polypeptides.

In our experiments gastrin, caerulein and desulphated caerulein, as well as ACh and CCK-PZ, all depolarized the pancreatic acinar cells of mouse and rat concomitant with reduced membrane input resistance. The depolarizing effect of ACh, but not that of other stimulants, was abolished by atropine, suggesting the existence of two distinct receptors for these stimulants. Furthermore, we can divide peptide agonists into two groups: the CCK-PZ group and the gastrin group. The first group requires the presence of a sulphated tyrosyl residue at position 7 from the C-terminus; the second group requires the presence of tetrapeptide amide. The CCK-PZ group has a stronger affinity for the receptor than the gastrin analogues. It was confirmed by the dose-response curve of these agonists on pancreatic acinar cells of mouse and rat that the biological activity was in the following order of potency: Cn > CCK-PZ > gastrin.

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# COMPARATIVE EFFECTS OF GLYCERYL TRINITRATE ON VENOUS AND ARTERIAL SMOOTH MUSCLE *in vitro;* RELEVANCE TO ANTIANGINAL ACTIVITY

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1 A quantitative *in vitro* study has been made of the actions of glyceryl trinitrate and sodium nitrite on vascular smooth muscle (dog femoral artery and saphenous vein; rat portal vein); these have been compared with the actions of papaverine, isoprenaline, salbutamol, pentaerythritol tetranitrate and trimetazidine.

2 Glyceryl trinitrate was more active on the saphenous vein than on the femoral artery in inhibiting noradrenaline and potassium-induced tone.

3 Unlike glyceryl trinitrate, sodium nitrite and isoprenaline, papaverine and diazoxide inhibited noradrenaline-induced contractions of venous and arterial smooth muscle to the same extent.

4 The selective dilator effects of glyceryl trinitrate on venous smooth muscle may explain its action in alleviating the pain of angina pectoris. It is suggested that the use of these three vascular smooth muscle preparations (arterial, and veins with and without spontaneous myogenic activity) is a useful initial screening procedure for prospective antianginal drugs acting by venodilatation.

## Introduction

It is nearly a century since William Murrell suggested that glyceryl trinitrate (nitroglycerin) would be effective in the treatment of angina pectoris. He deduced that 'from a consideration of the physiological action of the drug and more especially from the similarity existing between its general action and that of nitrite of amyl . . . it would probably prove of service in the treatment of angina pectoris' (Murrell, 1879). From his clinical experience he concluded, 'I am happy to say that this anticipation has been realised'. Like Lauder Brunton, Murrell felt that the main effect was on the peripheral vessels. Recent clinical studies have tended to confirm this conclusion. The abnormally increased left ventricular filling pressure, end-diastolic volume, ventricular dimensions and myocardial wall tension, which occur at the height of an anginal attack, are all reduced by nitroglycerin (Williams, Glick & Braunwald, 1965; Lee, Sung & Zaragoza, 1970; Parker, 1972). These effects can be explained (see reviews by Parker, 1972, and by Parratt, 1974; 1975) by dilatation of peripheral capacitance vessels.

If this is true, then it ought to be possible to show preferential dilator effects with nitroglycerin and related compounds on isolated venous, as compared to arterial, smooth muscle; it would then be feasible to screen large numbers of prospective antianginal drugs using simple, inexpensive, isolated vascular smooth muscle preparations. Such drugs, like nitroglycerin itself (Hirshfeld, Borer, Goldstein, Barrett & Epstein, 1974; Smith, Redwood, McCarron & Epstein, 1974) might also be effective in reducing the severity and extent of myocardial damage (infarction) which results from occlusion of a coronary artery. It was with these possibilities in mind that the following experiments were carried out.

## Methods

### *Isolated femoral artery and saphenous vein of the dog*

The preparations were obtained from anaesthetized greyhounds (20–35 kg) after experiments, at the Western Infirmary, involving the effect of hyperbaric oxygen on ischaemic cardiac muscle blood flow (Ledingham, Marshall & Parratt, unpublished). Lengths of femoral artery and saphenous veins, free from major branches, were removed and stored overnight in Krebs solution at 2–3°C. In order to examine the effects of drugs on isolated vascular smooth muscle, thin transverse sections were cut once to produce strips, each of which was suspended in a

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10 ml organ bath containing Krebs solution (composition: mM; NaCl 118, KCl 4.4, $NaH_2PO_4$ 25, $MgCl_2$ 1.25, $CaCl_2$ 5.4 and glucose 5.5) at 37°C. The solution was gassed with a mixture of 5% $CO_2$ in $O_2$. Contractions (of circular smooth muscle layers) were recorded isometrically with Ugo-Basile strain-gauges, Devices pre-amplifiers and a two-channel recorder. Arterial and venous preparations from the same dog were studied simultaneously. The resting tension was 0.5 gram. The femoral artery and saphenous vein strips had very little resting tone and were therefore contracted with either noradrenaline (2–10 µM) or potassium ($K^+$; 50–100 mM). Submaximal (60–80% of maximum) contractions induced by these agonists were maintained and reproducible. Sodium nitrite, glyceryl trinitrate and the other drugs mentioned in the Results section, were added to the bath in increasing concentrations, without washing, and the degree of inhibition of the noradrenaline or $K^+$-induced tone was assessed.

### *Isolated portal vein of the rat and dog*

Portal veins were removed from rats weighing between 200 and 500 g and were immersed in Krebs solution at a temperature of 37°C. A tension of 0.5 g was applied to the tissue and the contractions recorded isometrically. Longitudinal strips prepared from the portal vein of the dog were also used. Both preparations exhibited inherent, spontaneous, myogenic activity, and inhibition of this activity by the cumulative addition of various drugs was examined.

The drugs used were: alprenolol (Hassle), atropine, glyceryl trinitrate (Evans Medical), (—)-isoprenaline (Wyeth), (—)-noradrenaline (Sigma), salbutamol (Allen & Hanbury), sodium nitrite, pentaerythritol tetranitrate (Warner) and trimetazidine (Servier).

## Results

### *Effects of sodium nitrite*

Sodium nitrite relaxed all three types of vessels studied (Figure 1). The threshold concentration (100 µM), and the slope of the dose-response curves up to approximately 40% inhibition (approximately 1 mM; Figure 1) were similar. Concentrations in excess of 1 mM continued to inhibit the tone of the saphenous and portal veins up to 80% inhibition (10 mM; the highest concentration used in the rat portal vein). Complete inhibition of induced tone was produced in the femoral vein with 30 mM of sodium nitrite. In contrast to the effects on the venous preparations, there was no further reduction in arterial tone with increasing concentrations of sodium nitrite (from 1 to 10 mM); concentrations in excess of 10 mM increased the tone such that, at the highest concentration (100 mM), it was only 10% less than the original control level. An example of such an experiment is shown in Figure 2. This response is presumably due to the high concentration of sodium ions releasing calcium from intracellular stores.

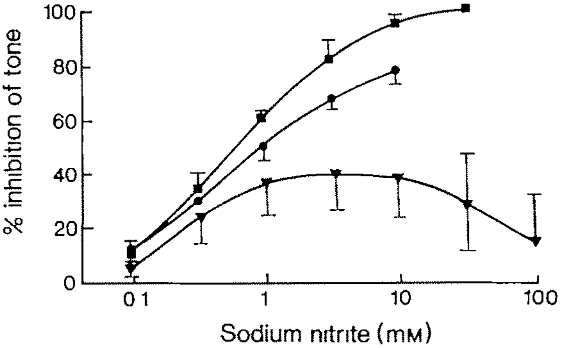

**Figure 1** The inhibitory effect of sodium nitrite on the inherent myogenic tone of the rat portal vein (●) and on tone induced by noradrenaline (2–10 µM) in the dog saphenous vein (■) and femoral artery (▼). Note the unusual shape of the dose-response curve for sodium nitrite on the femoral artery Each point represents the mean of five to six observations Vertical lines show s.e. mean.

### *Effects of glyceryl trinitrate*

Initially, glyceryl trinitrate solutions were made from tablets but in the later experiments dilutions were made, in distilled water, from a stock solution of glyceryl trinitrate contained in alcohol. Because alcohol itself relaxed the vascular smooth muscle preparations used, solvent controls were carried out in all experiments. No strength of glyceryl trinitrate was used which contained greater than one-tenth the concentration of solvent which itself produced a response. The results with glyceryl trinitrate obtained from both sources (tablets and alcoholic solution) were identical.

The responses to glyceryl trinitrate were similar to those of sodium nitrite (Figure 3). In the femoral vein complete inhibition was obtained (at 50 µM; five preparations) without approaching the threshold to the alcohol response. In contrast, the femoral artery could only be inhibited by about 50%, even with concentrations in excess of 5 mM. The threshold dose for the artery was about 0.4 µM, i.e. 10 times that for the vein (Figure 3).

In order to determine whether the glyceryl trinitrate-induced relaxation of the femoral vessels was due to a specific antagonism of noradrenaline, the experiments were repeated using potassium instead of noradrenaline to induce contracture. The threshold concentration of glyceryl trinitrate was similar (0.4 µM) in these experiments, with maximum inhibition (60%) of the artery occurring at a concentration of 50 µM (five preparations) and of the vein

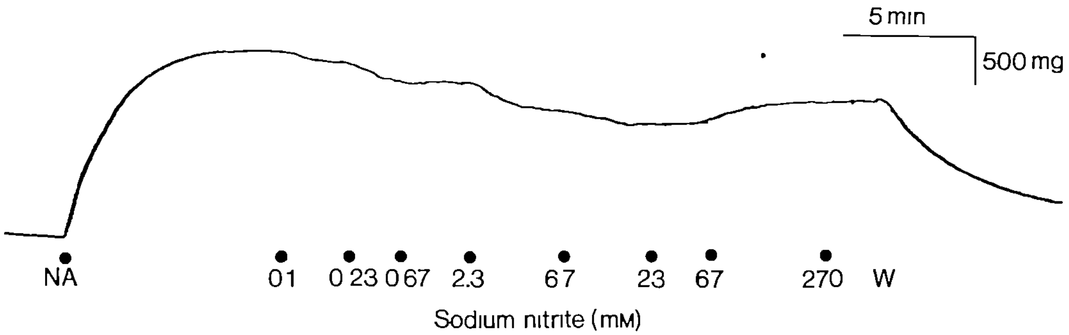

**Figure 2** The inhibitory effect of cumulative additions of sodium nitrite on the tone induced by noradrenaline (NA, 2 μM) in the dog femoral artery. Note the increase in tone produced by concentrations of sodium nitrite in excess of 100 μM. W=wash.

(100% inhibition) at 500 μM (six preparations). The degree of inhibition of the femoral vein (100%) and artery (55–60%) was the same against a potassium-induced contracture as against one induced by noradrenaline (Figure 3) although higher concentrations of glyceryl trinitrate had to be used.

Inhibition of noradrenaline-induced contraction by glyceryl trinitrate and sodium nitrite was unaffected by pretreatment with atropine (1.4 μM), alprenolol (1.8 μM) or mepyramine (3.5 μM). These concentrations were sufficient to antagonize the actions of acetylcholine, isoprenaline or histamine respectively.

*Effects of other vasodilator drugs*

The fact that sodium nitrite and glyceryl trinitrate preferentially relaxed venous smooth muscle could be explained by simple ease of drug access, in the thinner venous preparations, to the receptors involved. That this is not the explanation is clear from Figure 4 which shows the comparative effects of papaverine and diazoxide on saphenous vein and femoral artery preparations. Both drugs inhibited noradrenaline-induced contractions to the same extent in the two preparations.

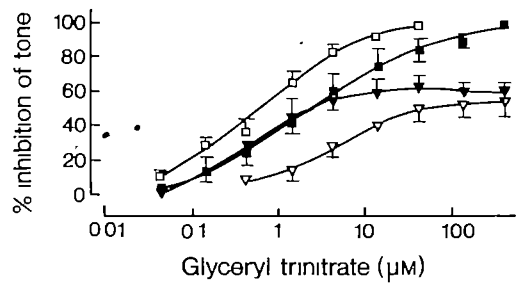

**Figure 3** A comparison of the effect of glyceryl trinitrate on tone induced by noradrenaline (open symbols) and by potassium (closed symbols) in the dog femoral artery (▽▼) and saphenous vein (□■). Each point represents the mean of at least five observations. Vertical lines show s.e. mean.

Some indication of the relative effectiveness of glyceryl trinitrate was obtained by comparison of its effects on all three preparations with those of isoprenaline (Figure 5) and, on the rat portal vein, with salbutamol and other antianginal agents, pentaerythritol tetranitrate and trimetazidine. The results were summarized in Figure 6.

## Discussion

The results obtained with the dog isolated vessels indicate that both glyceryl trinitrate and sodium nitrite

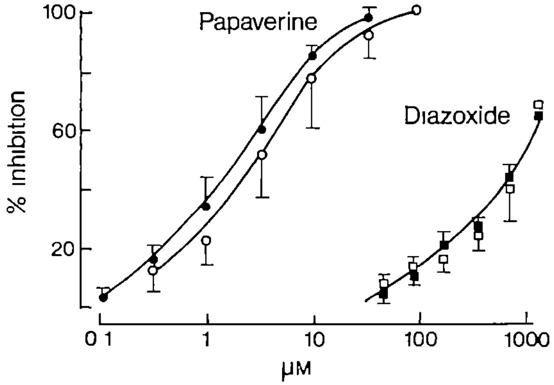

**Figure 4** Comparative effects of papaverine and of diazoxide on tone induced by noradrenaline in the dog femoral artery (○□) and saphenous vein (●■). Each point represents the mean of five to six observations. Vertical lines show s.e. mean. In contrast to the effect of glyceryl trinitrate, both these drugs inhibited arterial and venous smooth muscle preparations to the same extent, i.e. they showed no particular selectivity for venous smooth muscle.

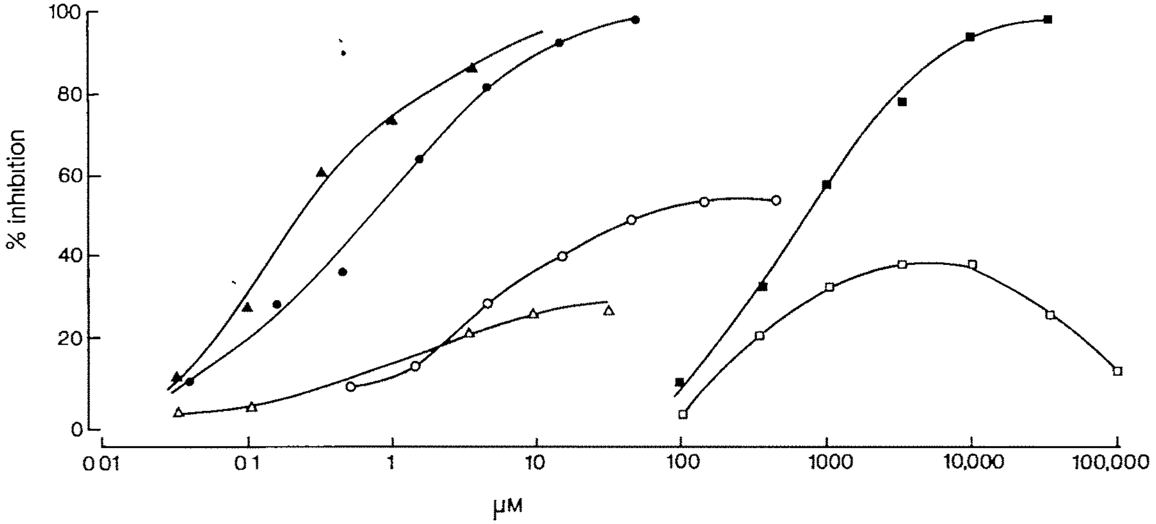

**Figure 5** The effects of isoprenaline (▲△) glyceryl trinitrate (●○) and sodium nitrite (■□) on dog saphenous veins (closed symbols) and femoral arteries (open symbols) contracted with noradrenaline (2–10 µM). Each point represents the mean of six observations. All three agents inhibit venous smooth muscle more than they do arterial smooth muscle, i e they demonstrate selectivity for venous smooth muscle.

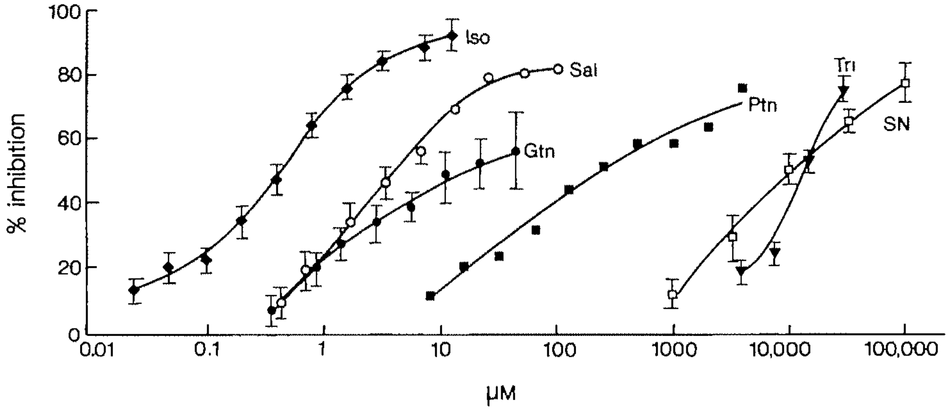

**Figure 6** Comparison of the effect of isoprenaline (Iso), salbutamol (Sal), glyceryl trinitrate (Gtn), pentaerythritol tetranitrate (Ptn), trimetazidine (Tri) and sodium nitrite (SN) on spontaneous myogenic activity of rat portal veins.

preferentially relax venous smooth muscle, especially when it is contracted with noradrenaline. For example, at a concentration of 1 µM glyceryl trinitrate inhibited, by more than 50%, noradrenaline-induced tone in the saphenous vein whereas this concentration had only a slight (<10%) effect on noradrenaline-induced tone in the femoral artery. At all concentrations glyceryl trinitrate was significantly ($P<0.001$) more effective on the saphenous vein than on the femoral artery. Even in concentrations in excess of 100 µM it could only inhibit tone in arterial preparations by 50%. In contrast, noradrenaline-induced tone in venous smooth muscle was completely inhibited by a concentration of only 10 µM. A similar differential effect on the two smooth muscle preparations was also obtained with isoprenaline, which was equiactive with glyceryl trinitrate, and with sodium nitrite, which was 1000 times less active. This indicates that nitroglycerin does not have to be reduced from the ester to the nitrite ion before it becomes an active venodilator and also that only relatively small amounts of circulating nitroglycerin are required to dilate veins in which tone is increased with noradrenaline. An increased sensitivity of peripheral veins to this neurotransmitter has been recently demonstrated in anginal patients by Robinson (1975).

This selectivity for venous smooth muscle is not due to the relative thinness of the walls of the veins allowing ease of drug access since papaverine and diazoxide showed no such selectivity for venous smooth muscle. In fact papaverine was active in a similar concentration to glyceryl trinitrate and isoprenaline on the noradrenaline-contracted saphenous vein but was much more active than either drug on the femoral artery.

The results obtained from these relatively simple *in vitro* experiments are in general agreement with those obtained with more sophisticated *in vivo* models. Using the Mellander (1960) technique for simultaneously assessing changes in the responses of series-coupled resistance and capacitance vessels, and on precapillary sphincter activity in the cat hindquarters, Johnsson & Öberg (1968) found that nitroglycerin and sodium nitrite had more marked effects on capacitance vessels. On the other hand, isoprenaline (Johnsson & Öberg, 1968) and hydralazine (Ablad & Mellander, 1963) had more pronounced dilator effects on precapillary resistance vessels; this, by decreasing the pre- to post-capillary resistance ratio, favoured transcapillary exchange. There are studies on the human forearm that also indicate that the main site of action of nitroglycerin is on capacitance vessels (Mason & Braunwald, 1965; Williams *et al.*, 1965). The resultant venous pooling and reduced return of blood to the right side of the heart, together with pulmonary vasodilatation (Cyong, Tanaka, Horiguchi, Tsuchiya & Itoh, 1976), would adequately explain the decreased ventricular filling pressure, end-diastolic volume and heart size, and the resultant reduction in myocardial oxygen consumption, reported to occur in anginal patients after nitroglycerin (see Introduction).

Whilst papaverine was able to abolish completely noradrenaline-induced tone in both arterial and venous tissues, the maximum inhibition of tone produced by glyceryl trinitrate was not the same, being much less in arterial than in venous smooth muscle (Figure 3). Furthermore this difference was independent of the means by which tone was induced (i.e. with noradrenaline or potassium, Figure 3). Similar degrees of inhibition were produced by isoprenaline (Figure 5). An additional similarity between isoprenaline and glyceryl trinitrate is that they are both potentiated by sub-inhibitory concentrations of phosphodiesterase inhibitors (Levy & Wilkenfeld, 1968). Isoprenaline relaxes vessels by $\beta$-adrenoceptor activation which results in stimulation of adenyl cyclase and the production of cyclic adenosine 3′,5′-monophosphate (cyclic AMP); this leads to $Ca^{2+}$ sequestration and relaxation (Andersson, 1973). Phosphodiesterase inhibitors potentiate this action of isoprenaline by preventing the breakdown of cyclic AMP to AMP. It has been shown that glyceryl trinitrate is not itself a phosphodiesterase inhibitor (Levy & Wilkenfeld, 1968; Poch & Kukovetz, 1972) and the present work shows that an effect on $\beta$-adrenoceptors is not involved since its actions were unaffected by alprenolol. The similarity between the two drugs could be explained by glyceryl trinitrate stimulating adenyl cyclase either directly, or by a process not involving $\beta$-adrenoceptors. The release of bound $Ca^{2+}$ by agonists would then be prevented as a result of the increased levels of cyclic AMP by an action of nitroglycerin at a site distal to the $\beta$-adrenoceptor but proximal to that of phosphodiesterase inhibition. The difference in maximum inhibition of veins and arteries could then be due to a difference in the maximum cyclic AMP levels attainable.

One approach to more effective anginal therapy is to produce drugs that have a similar basic mode of action to nitroglycerin. The present studies have emphasized that this mechanism is a selective dilator action on peripheral veins. We suggest therefore that a simple primary screen for new antianginal compounds would be to examine their relative activity on isolated arteries and veins (contracted with noradrenaline or potassium) and on isolated veins with spontaneous myogenic activity (e.g. portal vein of the rat). Such experiments would not of course give any indication of effects on the microcirculation; they should however indicate which compounds would be worth pursuing using more sophisticated tests for antianginal activity (Parratt, 1974).

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# THE EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON CHOLINERGIC AND HISTAMINE-INDUCED CONTRACTIONS OF GUINEA-PIG ISOLATED ILEUM

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1 Eleven non-steroidal anti-inflammatory drugs (NSAID) reversibly inhibited contractions of the longitudinal muscle of the guinea-pig isolated ileum induced by acetylcholine, histamine, electrical transmural stimulation and nicotine in this order of increasing potency.

2 After the addition of prostaglandins $E_1$, $E_2$ or $F_{2\alpha}$, with partially effective concentrations of NSAID (but not with higher concentrations which almost totally prevented the responses) the inhibitory effects of NSAID were reversibly lost, except for electrically induced contractions and prostaglandin $F_{2\alpha}$.

3 The effects of NSAID may be due to actions on biological membranes or on distribution of ions in addition to their inhibitory effect on prostaglandin synthesis. Prostaglandins may reverse the inhibition by non-selective sensitization of smooth muscle to various agonists.

## Introduction

Prostaglandins seem to play a role in contractions of intestinal smooth muscle (Bergström, Eliasson, Von Euler & Sjöval, 1959; Bennett, Eley & Scholes, 1968; Fleshler & Bennett, 1969; Eckenfels & Vane, 1972; Ferreira, Herman & Vane, 1976). Since the demonstration that indomethacin inhibits prostaglandin synthesis in cell-free homogenates of guinea-pig lung (Vane, 1971), many authors have confirmed the inhibitory action of non-steroidal anti-inflammatory drugs (NSAID) on prostaglandin synthesis in various tissues (Flower, 1974).

Ehrenpreis, Greenberg & Belman (1973) and Bennett, Eley & Stockley (1975) found that indomethacin (40 µg/ml) substantially reduced contractions of the longitudinal muscle of guinea-pig isolated ileum in Krebs solution to nerve stimulation with transmural electrical pulses. Kadlec, Mašek & Šeferna (1974) obtained similar results with indomethacin (0.36 µg/ml) in a modified Krebs solution. The difference in the concentration of indomethacin needed depends on the composition of the bathing solution (Bennett *et al.*, 1975). All these authors reversed the inhibitory action of indomethacin with low concentrations of prostaglandin E.

Ehrenpreis *et al.* (1973) concluded that prostaglandins couple cholinergic nerve terminal excitation with acetylcholine release while Bennett *et al.* (1975) thought that prostaglandins modulate the response to cholinergic activity. Kadlec *et al.* (1974) postulated that this prostaglandin-modulated action on cholinergic activity is mediated through inhibition of noradrenaline release.

Bennett *et al.* (1975) showed that indomethacin 2 µg/ml also greatly depressed nicotine-induced contractions of guinea-pig isolated ileum whereas 5–20 µg/ml caused little reduction in the contractions to exogenous acetylcholine and histamine (Chong & Downing, 1973; Bennett *et al.*, 1975). These inhibitory effects are reduced by small amounts of prostaglandins in the bath (Bennett *et al.*, 1975).

We have extended these observations to several other NSAID known to inhibit prostaglandin synthesis (Flower, 1974) in an attempt to understand better their mechanisms of action on intestinal smooth muscle.

## Methods

Adult guinea-pigs of either sex were stunned and bled. Segments of ileum (4 cm) at least 10 cm from the caecum were set up under an initial load of 1 g in Krebs-Henseleit solution (composition g/l : KCl 0.35, NaCl 6.92, $CaCl_2.2H_2O$ 0.37, $NaHCO_3$ 2.1, $KH_2PO_4$ 0.16, $MgSO_4.7H_2O$ 0.29 and glucose 1.0) at 37°C gassed with a mixture of 5% $CO_2$ and 95% $O_2$. Transmural stimulation was carried out as described by Paton (1955) with rectangular pulses of 0.5 ms duration, 0.1 Hz, 5 to 25 V delivered and measured by a Grass stimulator. Muscle contraction was registered isometrically by a transducer.

The inhibitory effect of 11 NSAID (indomethacin, flufenamic, mefenamic and niflumic acids, bufexamac, alclofenac, clopirac, ibuprofen, phenylbutazone, oxyphenbutazone and amidopyrine) were assessed on these electrically-induced contractions. Five different concentrations (2.5, 10, 40, 100 and 200 µg/ml) were chosen to be tested, but only 3 or 4 of them were used for each drug according to the degree of inhibition observed. At least 4 experiments were performed at each drug concentration. The results are expressed as a percentage of the contractions obtained before addition of the drug. After an inhibition was observed, small amounts of prostaglandins $E_1$ (2.5 ng/ml), $E_2$ (2.5 ng/ml) or $F_{2\alpha}$ (tromethamine salt, 25 ng/ml) were added to the bath 12 min after the NSAID addition, but reversal of inhibition was not measured.

The same 11 NSAID were tested in parallel (12 min contact time) for inhibitory effects on acetylcholine, histamine and nicotine-induced contractions, using the lowest concentration tested which reduced the electrically-induced contractions by at least 50%. Segments of ileum were set up in identical conditions except that contractions were recorded on a kymograph with an isotonic lever (five-fold magnification). The submaximal contractions induced in the presence of the NSAID by 30 ng/ml histamine and 0.5 µg/ml nicotine were recorded every 3 min and 6 min respectively. The results are expressed as a percentage of the average of at least 3 control responses obtained before addition of the NSAID. In 4 of the 8 experiments with each NSAID, prostaglandin $E_1$ (2.5 ng/ml) was added to the bath immediately after the first (nicotine) or second (histamine) evoked contraction following the NSAID addition (Figure 3). The height of the contractions in the presence of both prostaglandin $E_1$ and NSAID is expressed as a percentage of the average of at least 3 control contractions.

In each experiment the ileum was finally challenged with the agonist, after washing out the NSAID (and prostaglandin $E_1$ if present). The height of three consecutive contractions is expressed as a percentage of the control contractions.

In other similar experiments prostaglandins $E_2$ (2.5 ng/ml) and $F_{2\alpha}$ (tromethamine salt, 25 ng/ml) were tested in the presence of each NSAID but their effect was estimated quantitatively only for indomethacin, phenylbutazone (Figure 3) and flufenamic acid. The effects of prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ were similarly tested in 4 experiments for each prostaglandin after the second evoked contraction induced by acetylcholine (20 ng/ml). The NSAID inhibition of contractions to acetylcholine has been described previously (Famaey, Fontaine & Reuse, 1977).

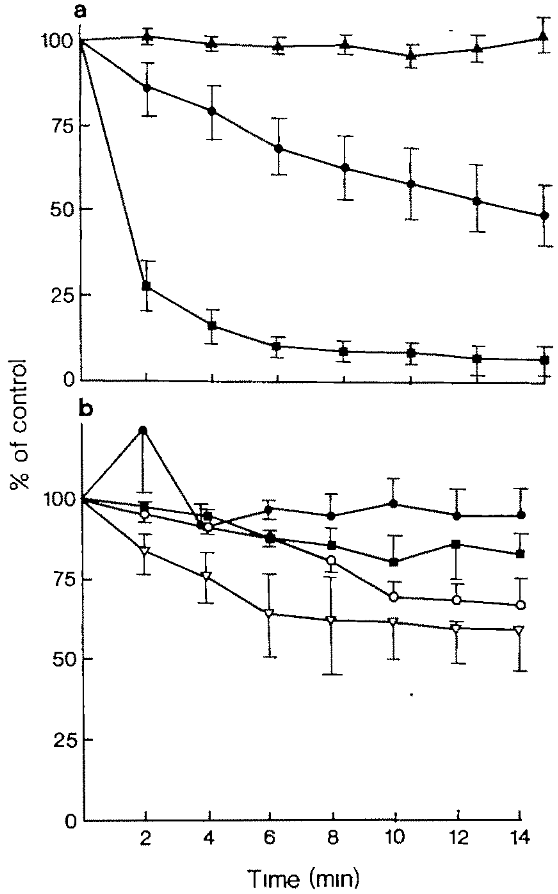

**Figure 1** The influence of drug contact time on the inhibition by various concentrations of (a) flufenamic acid and (b) alclofenac on the responses of guinea-pig ileum to electrical stimulation: (▲) 2.5 µg/ml; (●) 10 µg/ml; (■) 40 µg/ml; (○) 100 µg/ml and (▽) 200 µg/ml.

Results were analyzed by Student's *t* tests, except when differently quoted.

### *Drugs*

Gifts of the following drugs were obtained: prostaglandins $E_1$, $E_2$ and the tromethamine salt of prostaglandin $F_{2\alpha}$ (Upjohn Co, Kalamazoo, Michigan, U.S.A.), acetylcholine (Roche, Basel, Switzerland), phenylbutazone, oxyphenbutazone and amidopyrine (Ciba-Geigy, Basel, Switzerland), clopirac, bufexamac and alclofenac (Continental Pharma, Brussels, Belgium), ibuprofen (Boots Drug Co, Nottingham, Great Britain), indomethacin (Merck Sharp and Dohme, Rahway, New Jersey, U.S.A.), niflumic acid (Upsa, Agens, France), flufenamic and mefenamic

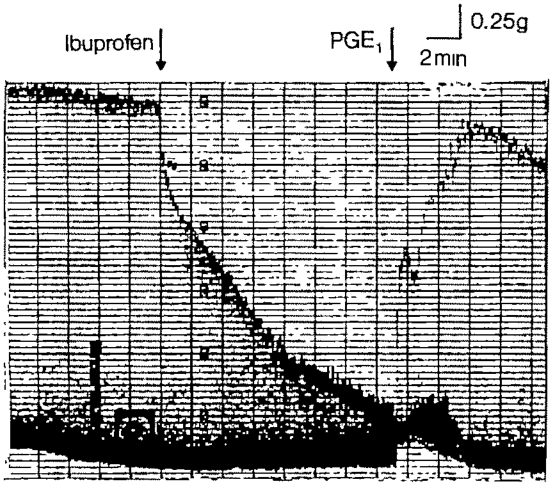

Figure 2 Almost total reversal by prostaglandin $E_1$ (PGE$_1$) 2.5 ng/ml of the inhibition induced by ibuprofen 40 µg/ml on responses of guinea-pig ileum to electrical stimulation.

acids (Parke Davis & Co, Detroit, Michigan, U.S.A.). Histamine was purchased from Fluka A.G. (Buchs, Switzerland) and nicotine sulphate from BDH. All other chemicals were of the purest grade commercially available.

## Results

All 11 NSAID tested caused a dose-related inhibition of the electrically-induced contractions of the guinea-pig ileum. The concentrations of NSAID used were 2.5–40 µg/ml for all drugs except phenylbutazone, oxyphenbutazone and alclofenac which required 10–200 µg/ml (Table 1, Figure 1). The effect increased progressively with the duration of contact and was maximum at about 12 min (Figure 1).

Prostaglandin $E_1$ or $E_2$ (2.5 ng/ml) reversed this submaximal inhibition, prostaglandin $E_1$ being somewhat more potent than prostaglandin $E_2$ (Figure 2), while prostaglandin $F_{2\alpha}$ (25 ng/ml) had little effect.

Prostaglandin E compounds clearly reversed a partial inhibition by NSAID but only slightly restored responses that had been almost abolished. The NSAID, at concentrations up to 200 µg/ml which induced at least 50% inhibition (with the exception of alclofenac) of the electrically-induced contractions after a 12 min contact time, also inhibited contractions induced by histamine or nicotine ($P<0.001$) (Figure 3). With every drug the inhibition of electrically-induced or nicotine-induced contractions was significantly higher than that of contractions to histamine (except for bufexamac, 10 µg/ml where inhibition of electrically-induced contraction was not significantly greater than that of histamine).

Indomethacin, flufenamic, mefenamic and niflumic acids, ibuprofen (all at 40 µg/ml) and bufexamac (40 and 10 µg/ml) inhibited nicotine-induced contractions significantly more than those to electrical stimulation.

Prostaglandin $E_1$ (2.5 ng/ml) completely reversed the NSAID-induced inhibition of contractions to histamine, nicotine and also to acetylcholine ($P<0.05$ to $P<0.001$ according to the drug and agonist—see Table 1) and even increased most of the contractions to above the control levels ($P<0.001$ for nicotine, $P<0.01$ for acetylcholine and $P<0.05$ for histamine). There were no significant differences between the contractions obtained after washing out the NSAID (and prostaglandin $E_1$ if present) and the average of the control contractions, demonstrating the reversibility of the NSAID and the prostaglandin $E_1$ effects.

Prostaglandin $E_2$ (2.5 ng/ml) and prostaglandin $F_{2\alpha}$ (25 ng/ml) gave similar results for all the NSAID and for each of the 3 agonists, to those obtained with prostaglandin $E_1$ and their statistical analysis for indomethacin, flufenamic acid and phenylbutazone also gave the same $P$ values.

As with electrically-induced contractions, prostaglandins markedly reversed submaximally inhibited contractions but had little effect on responses that had been totally inhibited (Table 1).

## Discussion

The inhibitory effect of indomethacin on electrically-induced contractions of the guinea-pig ileum described by Ehrenpreis *et al.* (1973), Kadlec *et al.* (1974) and Bennett *et al.* (1975) was confirmed by us and extended to 10 other acidic NSAID which inhibit prostaglandin synthesis (Flower, 1974) but have different chemical structures.

Inhibition of acetylcholine-induced contractions of guinea-pig ileum by indomethacin (Bennett *et al.*, 1975) and by the other NSAID (Famaey *et al.*, 1977) has been described and inhibition by these agents is now extended to histamine- and nicotine-induced contractions. With every NSAID the inhibition of electrically-induced or nicotine-induced contractions was significantly greater than the inhibition of contractions to acetylcholine. Only indomethacin, flufenamic and niflumic acids, clopirac (all at 40 µg/ml) and bufexamac (10 µg/ml) inhibited histamine-induced contractions significantly more than those to acetylcholine. The comparison between the averages of the mean inhibition obtained with each drug for each of the four agonists shows that NSAID inhibit the contractions to histamine and to acetylcholine significantly less than those to nicotine or to electrical stimulations ($P<0.001$, Mann Whitney U

**Table 1** A statistical analysis of the inhibitory effects of 11 non-steroidal anti-inflammatory drugs (NSAID) on guinea-pig ileum contractions induced by 4 different agonists and of the reversal by prostaglandin E$_1$ of these inhibitions

| *NSAID* | *µg/ml* | *Stim* | *ACh (Famaey Fontaine & Reuse, 1977)* | *ACh* + PGE$_1$ | *Hist* | *Hist* + PGE$_1$ | *Nic* | *Nic* + PGE$_1$ | *Results of Student's t tests* | |
|---|---|---|---|---|---|---|---|---|---|---|
| Indomethacin | 40 | 40.2±10.3 | 77.8±7.6 | 122.4±10.4<br><0.01 | 55.2±4.7 | 109.8±23.7<br><0.05 | 1.9±1.4 | no increase | (1)<0.05<br>(2)<0.05<br>(3)<0.001 | (4)<0.05<br>(5)<0.001<br>(6)<0.01 |
| | 10 | 76.8±11.9 | – | – | – | – | 40.7±5.7 | 174.9±27.8<br><0.001 | | |
| Flufenamic acid | 40 | 8.1±2.8 | 64.9±8.5 | 82.3±14.0<br><0.05 | 33.2±7.4 | 78.2±12.9<br><0.02 | 0 | no increase | (1)<0.001<br>(2)<0.02<br>(3)<0.001 | (4)<0.05<br>(5)<0.01<br>(6)<0.01 |
| | 10 | 64.0±10.1 | – | – | – | – | 18.2±2.8 | 178.2±29.3<br><0.001 | | |
| Mefenamic acid | 40 | 33.0±11.4 | 79.8±5.3 | 110.0±3.0<br><0.02 | 70.7±7.7 | 103.0±10.7<br><0.05 | 1.0±0.5 | no increase | (1)<0.01<br>(2) NS<br>(3)<0.001 | (4)<0.02<br>(5)<0.01<br>(6)<0.02 |
| | 10 | 59.6±8.0 | – | – | – | – | 33.8±7.0 | 191.6±16.2<br><0.001 | | |
| Niflumic acid | 40 | 29.6±7.2 | 93.3±3.3 | 133.2±15.7<br><0.01 | 71.2±5.4 | 173.2±44.9<br><0.01 | 10.1±4.1 | no increase | (1)<0.001<br>(2)<0.01<br>(3)<0.001 | (4<0.01<br>(5)<0.001<br>(6)<0.01 |
| | 10 | 92.5±6.8 | – | – | – | – | 70.1±12.4 | 205.7±28.6<br><0.01 | | |
| Bufexamac | 40 | 4.6±1.1 | 43.2±9.4 | 83.5±8.0<br><0.05 | 36.4±10.1 | 71.4±6.5<br><0.01 | 0 | no increase | (1)<0.02<br>(2) NS<br>(3)<0.01 | (4)<0.05<br>(5)<0.01<br>(6)<0.001 |
| | 10 | 43.0±7.3 | 66.0±5.7 | 142.5±5.7<br><0.05 | 46.7±12.9 | 82.9±6.8<br><0.01 | 15.7±3.8 | 106.3±10.8<br><0.01 | (1)<0.05<br>(2)<0.05<br>(3)<0.01 | (4) NS<br>(5)<0.05<br>(6)<0.01 |
| | 2.5 | 88.3±6.5 | — | — | — | — | 44.5±7.2 | 161.6±12.7<br><0.001 | | |
| Alclofenac | 200 | 80.2±14.4 | 97.0±2.5 | 150.7±11.0<br><0.001 | 96.4±2.4 | 163.3±18.7<br><0.001 | 74.9±5.7 | 249.7±36.1<br><0.001 | (1)<0.01<br>(2) NS<br>(3)<0.01 | (4)<0.01<br>(5)<0.01<br>(6) NS |
| Clopirac | 40 | 41.5±11.5 | 79.5±5.4 | 204.4±33.3<br><0.001 | 66.9±3.3 | 163.6±39.4<br><0.001 | 37.1±4.2 | 191.5±29.8<br><0.001 | (1)<0.01<br>(2)<0.05<br>(3)<0.001 | (4)<0.05<br>(5)<0.01<br>(6) NS |
| Ibuprofen | 40 | 39.5±7.0 | 76.6±5.5 | 191.3±9.2<br><0.001 | 72.8±7.8 | 151.4±4.2<br><0.01 | 22.0±2.6 | 132.4±3.1<br><0.001 | (1)<0.01<br>(2) NS<br>(3)<0.001 | (4)<0.02<br>(5)<0.001<br>(6)<0.02 |
| Phenylbutazone | 200 | 44.7±10.0 | 74.3±6.8 | 121.4±14.0<br><0.05 | 66.0±2.7 | 116.3±6.4<br><0.02 | 32.9±3.0 | 167.2±23.8<br><0.001 | (1)<0.05<br>(2) NS<br>(3)<0.01 | (4)<0.02<br>(5)<0.01<br>(6) NS |
| Oxyphenbutazone | 200 | 36.3±4.5 | 74.5±8.8 | 120.9±18.6<br><0.01 | 79.3±5.1 | 151.8±8.6<br><0.01 | 31.9±8.0 | 140.1±11.8<br><0.001 | (1)<0.05<br>(2) NS<br>(3)<0.02 | (4)<0.01<br>(5)<0.001<br>(6) NS |
| Amidopyrine | 40 | 36.0±14.5 | 86.4±8.1 | 126.7±10.8<br><0.05 | 76.8±6.8 | 109.7±3.0<br><0.05 | 45.1±4.6 | 159.5±24.0<br><0.001 | (1)<0.01<br>(2) NS<br>(3)<0.001 | (4)<0.01<br>(5)<0.001<br>(6) NS |

Stim = electrical stimulations; ACh = acetylcholine, Hist = histamine; Nic = nicotine

Prostaglandin E$_1$ (PGE$_1$) was used at the concentrations given in the Methods section The results are expressed as percentages of control contractions ± s.e. mean.

The statistical significance of the reversing effects of prostaglandins was established by comparison with the results obtained from the same agonist in the presence of the same NSAID alone. Student's *t* tests refer to (1) acetylcholine compared with electrical stimulation, (2) acetylcholine compared with histamine; (3) acetylcholine compared with nicotine; (4) histamine compared with electrical stimulation, (5) histamine compared with nicotine, (6) nicotine compared with electrical stimulation. NS = not significant

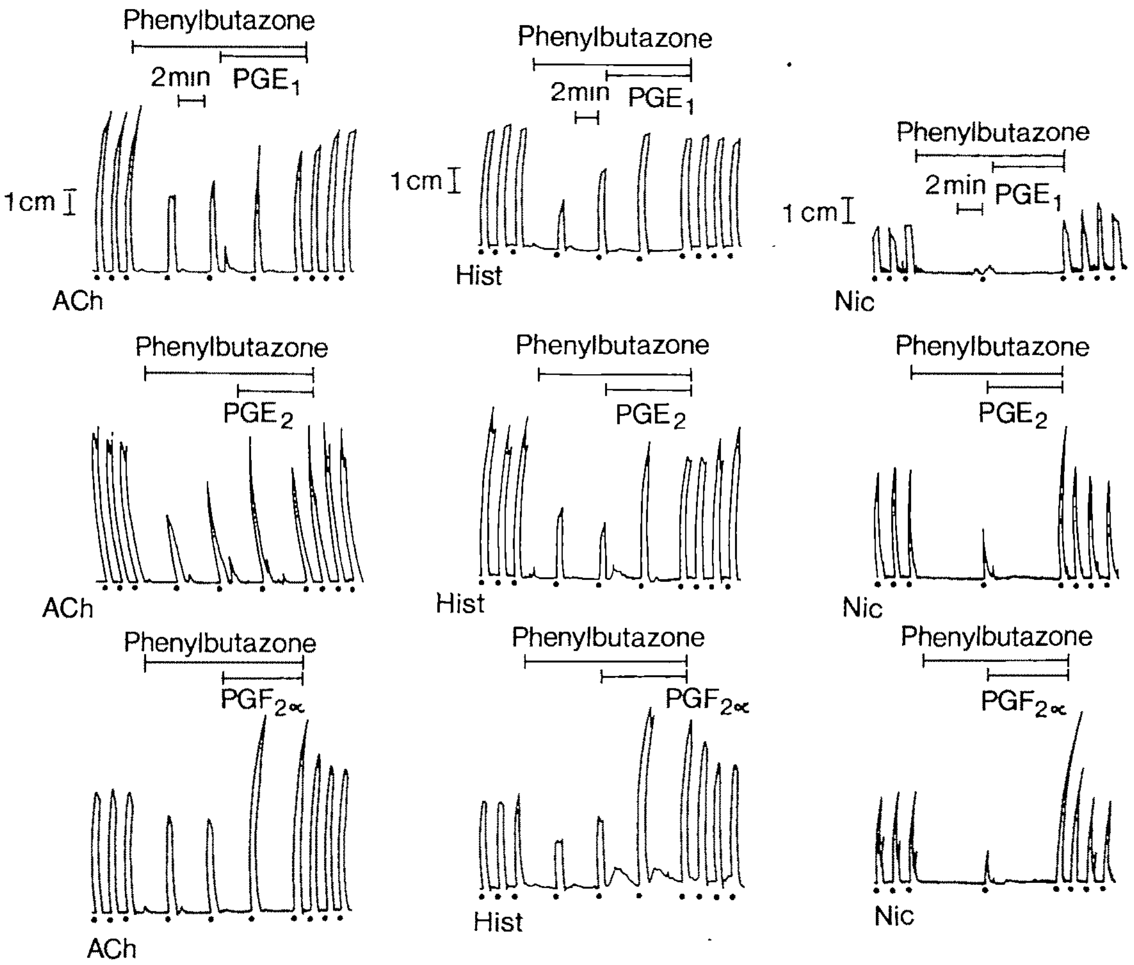

**Figure 3** The effect of phenylbutazone 200 µg/ml on guinea-pig ileum contractions induced at ● by acetylcholine (ACh), histamine (Hist) and nicotine (Nic) and the reversing effects of prostaglandin E$_1$ (PGE$_1$, 2.5 ng/ml) PGE$_2$ (2.5 ng/ml) and PGF$_{2\alpha}$(25 ng/ml).

test) and that there is no significant difference between the inhibitory effects of NSAID on contractions to acetylcholine or to histamine and between those on contractions to nicotine or electrical stimulations.

As suggested by Ehrenpreis *et al.* (1973) the greater inhibition of contractions to nicotine or electrical stimulation (indirect cholinergic effects) than to acetylcholine or histamine (direct agonists) could be explained by interference with neuronal acetylcholine release. However, nicotine which acts on ganglia (Day & Vane, 1963) was significantly more affected by NSAID than was the response to electrical stimulation which involves post-ganglionic cholinergic nerves (Paton, 1955). This suggests that the NSAID could be exerting some ganglion blocking effect on the guinea-pig isolated ileum. Ganglion blocking effects have not been clearly demonstrated for NSAID in other *in vitro* or *in vivo* models. Although Bennett *et al.* (1975) have obtained with indomethacin complete inhibition of nicotine-induced contractions and only partial inhibition of electrically-induced contractions, they did not point out any statistical difference between these two activities.

Our results show that all NSAID tested possess some spasmolytic activity as they all slightly inhibit contractions to histamine and to a lesser extent to acetylcholine. Others using similar concentrations, have demonstrated spasmolytic properties of several NSAID on gastrointestinal and vascular smooth muscles (Wilhelmi, 1949; Northover, 1967; Yamatake, Kato & Takagi, 1975).

We conclude that, in addition to effects which may be mediated by reduced acetylcholine release, similar to those previously described for indomethacin by Ehrenpreis *et al.* (1973), Kadlec *et al.* (1974) and Bennett *et al.* (1975), the NSAID exhibit on the guinea-pig isolated ileum slight spasmolytic, moderate antihistaminic and perhaps some ganglion blocking properties.

Several NSAID possess some antihistaminic properties (Domenjoz, 1960; Parrot, Ruff & Saindelle, 1966). Histamine is thought to be an important mediator in the inflammatory process (Schayer, 1962; Spector & Willoughby, 1964). Most of the NSAID are able to reduce oedema induced experimentally by histamine or histamine-releasers although to a lesser degree than true antihistaminic drugs (Winter, 1966). This is in accordance with our results which indicate that NSAID have only moderate antihistaminic properties.

We found that prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ completely reversed the submaximal inhibitory effect of NSAID on contractions to acetylcholine, histamine, nicotine and electrical stimulation (except for prostaglandin $F_{2\alpha}$ and electrical stimulation). Since NSAID inhibit prostaglandin synthesis at even lower concentrations than those used in our experiments (Vane, 1971), prostaglandins might be important for (1) the activation of ganglia by nicotine, (2) the release of acetylcholine by nerve stimulation, and (3) the contraction of smooth muscle by acetylcholine or histamine. Ehrenpreis *et al.* (1973) suggested this hypothesis for acetylcholine release by electrical nerve stimulation and extended it to the concept that morphine affects gastrointestinal motility by inhibiting prostaglandin-synthetase.

Another explanation was proposed by Bennett *et al.* (1975) who thought that prostaglandins might be involved in ileal contractions mediated by cholinergic nerves by increasing the response to released acetylcholine and possibly its amount. Prostaglandins might modulate responsiveness to other stimuli since they also reverse the inhibition of ileal histamine-induced contractions by NSAID. Eckenfels & Vane (1972) observed that following pretreatment of the guinea-pig colon with indomethacin, the initial contractor effect of histamine was unaffected but the contraction was not maintained unless prostaglandin $E_2$ was added.

Prostaglandins therefore seem to sensitize guinea-pig ileum non-selectively to all stimuli. This would explain why prostaglandins reverse NSAID inhibition and even induce contractions above control levels.

These NSAID effects on guinea-pig isolated ileum probably involve actions apart from inhibition of prostaglandin synthesis. At concentrations similar to those used in our experiments these drugs also inhibit various proteolytic enzymes, uncouple mitochondrial oxidative phosphorylation and interfere with various biological membranes (lysosomes, mitochondria, erythrocytes and lymphocytes) as well as non-biological membranes (Famaey, Brooks & Dick, 1975). These membrane properties are related to movements of various ions including cellular uptake or release of sodium, potassium or calcium (Northover, 1971; Famaey & Whitehouse, 1976).

Preliminary data indicate that most of the anti-inflammatory steroids and chloroquine which also affect membranes (Weissman, 1964) have very similar effects on contractions of guinea-pig ileum (Famaey, Fontaine, & Reuse, 1975; Famaey *et al.*, 1977). However anti-inflammatory steroids with no known inhibitory effects on prostaglandins synthesis in crude homogenates (Flower, 1974) might affect the release (Gryglewski, Panczenko, Korbut, Grodzinska & Ocetkiewicz, 1975; Lewis & Piper, 1975) as well as the production of prostaglandins by entire cell preparations (Kantrowitz, Robinson, MacGuire & Levine, 1975; Tashjian, Voelkel, MacDonough & Levine, 1975).

In conclusion it seems that NSAID are able to inhibit, in a dose-dependent manner, the contractions induced by various agonists, probably by inhibiting ileal prostaglandin synthesis and by affecting membrane properties and altering their permeability to various ions. The reversal effect observed in the presence of prostaglandins must be related to this inhibition of synthesis but may also be related to a non-specific ileal sensitization by prostaglandins to the various agonists tested.

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# TISSUE BLOOD FLOW AND DISTRIBUTION OF CARDIAC OUTPUT IN CATS: CHANGES CAUSED BY INTRAVENOUS INFUSIONS OF HISTAMINE AND HISTAMINE RECEPTOR AGONISTS

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1 The effects of infusions of histamine on blood pressure, cardiac output, heart rate, total peripheral resistance, stroke volume and tissue blood flow have been determined in anaesthetized cats using radio-active microspheres to measure cardiac output and tissue blood flow.

2 Histamine caused dose-dependent falls in blood pressure and total peripheral resistance over the dose-range $1 \times 10^{-8}$ to $3.3 \times 10^{-7}$ mol $kg^{-1}$ $min^{-1}$. Histamine had no effect on cardiac output, heart rate or stroke volume.

3 Histamine caused vasodilatation in the heart and stomach, with increased blood flow through these organs, and in the small and large intestine where blood flow was maintained despite the falls in arterial blood pressure. Blood flow to the brain, kidneys, liver, adrenal glands, skeletal muscle, spleen and skin was reduced when arterial blood pressure fell. Vascular resistance increased in the skin and spleen, presumably due to reflex vasoconstriction when blood pressure fell.

4 The selective $H_1$-receptor agonist 2-(2-aminoethyl)pyridine lowered blood pressure and decreased total peripheral resistance but did not change cardiac output, heart rate or stroke volume. 2-(2-Aminoethyl)pyridine caused vasodilatation in the heart, small and large intestine and kidneys. Vascular resistance was increased in the spleen and skin.

5 The selective $H_2$-receptor agonist 4-methylhistamine also lowered blood pressure and decreased total peripheral resistance but did not change cardiac output, heart rate or stroke volume. 4-Methylhistamine caused vasodilatation in the heart, stomach, small and large intestines and skeletal muscle. Vascular resistance was increased in the skin.

## Introduction

Dale and his colleagues (Dale & Laidlaw, 1910; 1919; Dale & Richards 1918) first described the potent depressor activity of histamine. The falls in blood pressure which followed histamine administration were associated with vasodilatation and presumably, a fall in total peripheral resistance. Subsequently, many studies have been made to investigate further the vasodilator properties of histamine, and vasodilatation has been studied after intra-arterial injection of histamine in many tissues including skeletal muscle (Haddy, 1960; Kjellmer & Odelram, 1965; Flynn & Owen, 1975), intestine (Lee, 1957, Flynn & Owen, 1975), kidney (Sinclair, Bell & Keyl, 1974) and the internal and external carotid beds (Tindall & Greenfield, 1973; Saxena, 1975).

In contrast, little is known about the effects of intravenous histamine on cardiac output and its distribution. Large doses of histamine, adequate to induce circulatory collapse, have been reported to decrease cardiac output (Blalock & Levy, 1937) whereas smaller doses may increase cardiac output (Delaunois, Kordecki, Polet & Ryzewski, 1959; Tucker, Weir, Reeves & Grover, 1975).

We have measured the effects of a range of doses of histamine, given by intravenous infusion, on blood pressure, cardiac output, total peripheral resistance and tissue blood flow. We have also taken the opportunity to compare the effects of histamine with those of a relatively selective histamine $H_1$-receptor agonist, 2-(2-aminoethyl)pyridine (Durant, Ganellin & Parsons, 1975) and a relatively selective histamine $H_2$-receptor agonist, 4-methylhistamine (Black, Duncan, Durant, Ganellin & Parsons, 1972). A preliminary account of part of this work has been presented to the British Pharmacological Society (Johnston & Owen, 1975).

## Methods

Simultaneous measurements of cardiac output and blood flow to major organs can be achieved by use of radio-active microspheres. The principles and validation of the technique have been described in detail elsewhere (for example Rudolph & Heymann, 1967; Neutze, Wyler & Rudolph 1968; Wagner, Rhodes, Sasaki & Ryan, 1969; Warren & Ledingham, 1974; Johnston, 1975).

Cats of either sex (weight 1.2–4.0 kg) were anaesthetized by an intraperitoneal injection of chloralose, 60 mg/kg, and urethane, 700 mg/kg. The trachea was cannulated. Blood pressure was measured from the right brachial artery with a Statham P23A blood pressure transducer and monitored on a Devices M2 electronic recorder. Heart rate was measured from the blood pressure trace. A catheter was placed in the left ventricle of the heart via the right carotid artery to allow injection of microspheres into the heart. A second catheter was tied into the right femoral artery for withdrawal of blood at a known rate (5 ml/min) during the injection of microspheres.

Ten minutes after completion of the surgical procedures, when blood pressure and heart rate were stable, approximately 100,000 $^{46}$Sc-labelled microspheres (25 µm diameter, 3M Company, Loughborough) were injected into the heart in order to measure resting cardiac output and tissue blood flows. Subsequently, intravenous infusions of 0.9% w/v NaCl solution (saline) (control experiments), histamine, 2-(2-aminoethyl)pyridine or 4-methylhistamine were given at a rate of 0.2 ml/minute. Thirty minutes later, and with the infusion still running, cardiac output and tissue blood flows were again measured with $^{85}$Sr-labelled microspheres. The animal was then killed by an overdose of sodium pentobarbitone and the organs of interest dissected out, weighed and their radioactivity measured in a Nuclear Enterprises 8312 Detector. The total radioactivity was measured in all organs except skin and skeletal muscle. Samples of skin were taken from the hindquarters and skeletal muscle from the abdominal wall and the left leg. Control experiments were done in 8 cats; 6 cats per group were used for each dose of histamine and the histamine-like agonists.

In all experiments the radioactivity in the lungs was determined. The mean proportion of microspheres in the lungs was 4.68 ± 0.4% ($n = 30$). This would include nutritional flow to the tissue and any microspheres which did not trap in the first capillary bed downstream from the site of injection. The low proportion of microspheres trapped in the lung indicates little shunting of blood through non-capillary beds in the peripheral circulation. In one cat the proportion of microspheres found in the lung exceeded 30%. Data from this animal have not been included.

The homogeneity of distribution of microspheres is indicated by the close correlation between blood flows to the right and left kidneys ($r = 0.96$, $n = 30$).

### *Calculations*

Cardiac output was calculated in ml/min from the number of microspheres in the blood withdrawn from the femoral artery at 5 ml/minute. Tissue blood flows were calculated from knowledge of cardiac output and the distribution of microspheres to tissues. Details of the calculations have been described by Johnston (1975).

Tissue blood flows were calculated in ml min$^{-1}$ 100g$^{-1}$ to provide values of flow per unit weight.

Tissue vascular resistance was calculated by dividing mean arterial blood pressure, mmHg, by tissue blood flow, ml min$^{-1}$ 100g$^{-1}$.

Values shown are mean ± s.e. mean. Significant changes in any parameter between control values and values measured during treatment periods were determined using a paired Student's $t$ test. Comparisons across groups were made using an unpaired Student's $t$ test.

### *Drugs*

Histamine acid phosphate (BDH), 2-(2-aminoethyl)pyridine dihydrochloride and 4-methylhistamine dihydrochloride were prepared as solutions in saline.

## Results

### *Tissue blood flow and distribution of cardiac output in cats*

The mean values of tissue blood flows and the distribution of cardiac output to tissues are shown in Table 1. The blood flow to the liver exceeds the flow to any other single organ and accounts for about 20% of the total cardiac output. Blood flow per unit weight was greatest in the adrenal glands, kidneys and heart although the adrenal glands are so small that they take only 0.5% of cardiac output despite their high flow per unit weight.

### *Effect of intravenous infusions of saline*

In eight control experiments the effects of a 30 min infusion of saline were determined. There were no changes in blood pressure or heart rate but a significant fall in cardiac output due to a fall in stroke volume. The fall in cardiac output with unchanged blood pressure led to a significant increase in calculated total peripheral resistance (Figure 1).

Blood flows to the brain, heart, kidneys and liver were well maintained with small falls in blood flow to the stomach, skin and intestines. In the small intestine, blood flow fell from $44.9 \pm 7.0$ to $34.8 \pm 4.3$ ml min$^{-1}$ 100g$^{-1}$. This fall was statistically significant ($P < 0.05$). Blood flow values during saline infusion are shown in Table 2. There were no significant changes in calculated tissue vascular resistance.

### *Effect of intravenous infusions of histamine*

Intravenous infusions of histamine caused dose-dependent falls in blood pressure and total peripheral resistance. The falls in blood pressure were statistically significant over the histamine dose range $3.3 \times 10^{-7}$ to $3.3 \times 10^{-8}$ mol kg$^{-1}$ min$^{-1}$. The falls in total peripheral resistance were significant at 1 and $3.3 \times 10^{-7}$ mol kg$^{-1}$ min$^{-1}$. Stroke volume and hence cardiac output were reduced at all histamine infusion rates but the reductions were similar to those in the saline-treated group. Heart rate was unchanged by histamine infusions. These changes are shown in Figure 1.

The effects of histamine on regional blood flow varied substantially from tissue to tissue and are summarized in Table 2. The calculated changes in vascular resistance associated with these flow changes are summarized in Table 3. The proportion of microspheres in the lungs was not significantly changed by any infusion rate of histamine. This would indicate that the proportion of microspheres not trapped in capillary beds but able to shunt through larger vessels was not changed by histamine.

Histamine significantly increased coronary blood flow and decreased coronary vascular resistance at all histamine infusion rates. Coronary vasodilatation was the only significant response to histamine observed at the lowest infusion rate. The maximum increase in coronary blood flow during infusions of histamine was approximately 2-fold, the maximum fall in vascular resistance was 76%.

Histamine also increased blood flow to the stomach and decreased gastric vascular resistance. These responses to histamine showed poor dose-dependence, significant changes in both parameters occurring only at the two higher histamine dose-levels. The maximum increase in gastric blood flow, during histamine infusions at $1 \times 10^{-7}$ mol kg$^{-1}$ min$^{-1}$, was almost 4-fold, the maximum fall in vascular resistance was 86%.

Histamine significantly increased renal blood flow during infusions at $3.3 \times 10^{-8}$ mol kg$^{-1}$ min$^{-1}$ and significantly decreased renal blood flow during infusions at 1 and $3.3 \times 10^{-7}$ mol kg$^{-1}$ min$^{-1}$. Renal vascular resistance was significantly reduced during infusions at $3.3 \times 10^{-8}$ mol kg$^{-1}$ min$^{-1}$.

Blood flow to the brain, liver and adrenal glands was not changed at the lower histamine infusion rates but significantly reduced at 1 and $3.3 \times 10^{-7}$ mol kg$^{-1}$ min$^{-1}$. Vascular resistance in these tissues was not significantly altered by histamine and the reduced blood flow at the higher infusion rates reflected the falls in mean arterial blood pressure at these higher histamine doses.

Blood flow to skeletal muscle tended to be reduced during infusion of histamine although none of the changes was statistically significant. Skeletal muscle vascular resistance was not significantly changed during infusion of histamine.

Skin blood flow was significantly reduced during infusion of histamine $3.3 \times 10^{-8}$ to $3.3 \times 10^{-7}$ kg$^{-1}$ min$^{-1}$, the maximum reduction exceeding 80%. Skin vascular resistance was significantly increased during infusion at 1 and $3.3 \times 10^{-7}$ mol kg$^{-1}$ min$^{-1}$, the maximum increase exceeding 4-fold. Histamine also significantly reduced splenic blood flow (maximum decrease 90%) and increased splenic vascular resistance (maximum increase 7-fold) during infusion at 1 and $3.3 \times 10^{-7}$ mol kg$^{-1}$ min$^{-1}$.

Blood flow to the small and large intestine was essentially unchanged by histamine. Blood flow to the

**Table 1** Tissue blood flow in anaesthetized cats ($n = 30$)

| *Tissue* | *Blood flow (ml/min)* | *(ml min$^{-1}$ 100 g$^{-1}$)* | *% cardiac output* |
|---|---|---|---|
| Brain | 17.9 ± 0.9 | 66.3 ± 3.1 | 5.4 ± 0.3 |
| Heart | 22.8 ± 1.1 | 306.1 ± 14.9 | 6.7 ± 0.3 |
| Kidneys | 40.0 ± 2.4 | 326.6 ± 14.9 | 14.4 ± 0.9 |
| Stomach | 4.9 ± 0.7 | 33.2 ± 4.5 | 1.4 ± 0.2 |
| Gastric mucosa | 3.2 ± 0.4 | 59.2 ± 10.3 | 0.9 ± 0.1 |
| Small intestine | 37.8 ± 3.4 | 50.8 ± 4.0 | 10.4 ± 0.6 |
| Large intestine | 12.4 ± 1.1 | 68.5 ± 5.8 | 3.5 ± 0.3 |
| Liver** | 69.8 ± 6.2 | 109.2 ± 9.9 | 20.2 ± 1.4 |
| Adrenal glands | 1.6 ± 0.1 | 477.4 ± 34.6 | 0.5 ± 0.03 |
| Spleen | 10.8 ± 1.5 | 206.9 ± 25.0 | 2.9 ± 0.3 |
| Skeletal muscle | – | 4.1 ± 0.3 | – |
| Skin | – | 5.3 ± 0.5 | – |

* Arterial blood flow only.

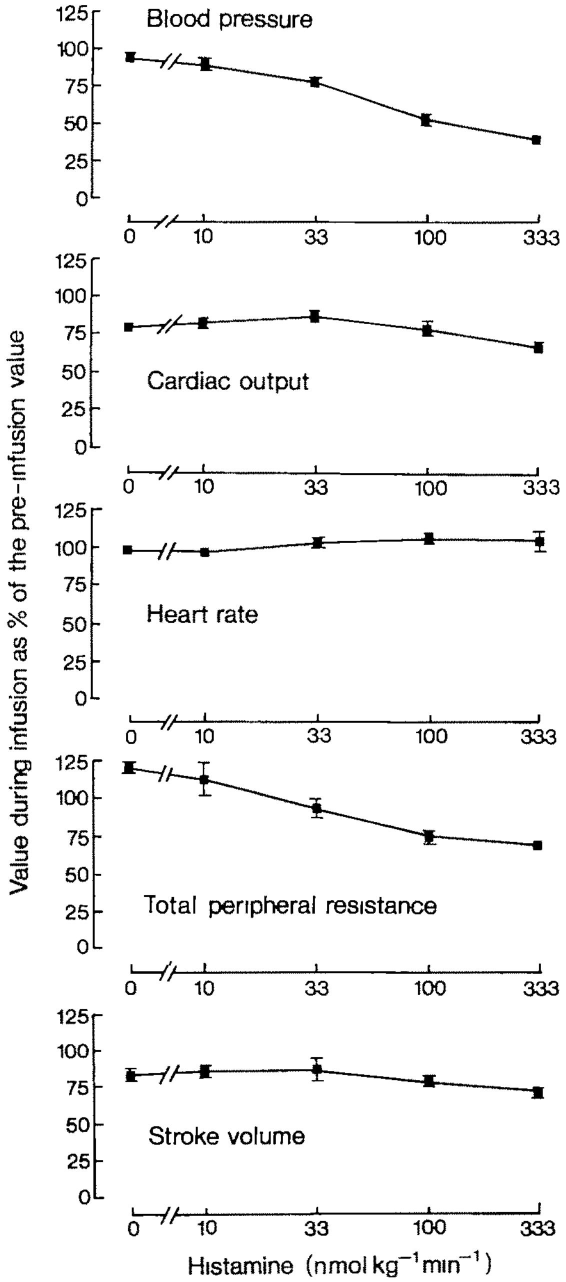

**Figure 1** Anaesthetized cats. Comparison of the effects of histamine with the effects of saline (shown as '0') on blood pressure, cardiac output, heart rate, total peripheral resistance and stroke volume. Values during infusions are expressed as a % of the pre-infusion control value. Vertical lines show s.e. mean. Histamine caused dose-dependent decreases in blood pressure and total peripheral resistance but did not change heart rate. Cardiac output and stroke volume fell in all groups including the group infused with saline.

intestine tended to fall during infusions of saline and similar falls occurred during the infusion of histamine 1 and 3.3 x $10^{-8}$ mol $kg^{-1}$ $min^{-1}$ whereas at the higher histamine infusion rates blood flow to the intestine was unchanged. Histamine significantly reduced intestinal vascular resistance during infusion at 1 and 3.3 x $10^{-7}$ mol $kg^{-1}$ $min^{-1}$.

The changes in tissue blood flow were sufficient to cause a redistribution of cardiac output. Blood flow to the heart increased from 7.9 ± 0.7% in saline-treated cats to 23.1 ± 1.1% during infusion of histamine, 1 x $10^{-7}$ mol $kg^{-1}$ $min^{-1}$. There was also a significant increase in distribution of cardiac output to the stomach; the proportions of cardiac output distributed to the brain, kidneys, liver, spleen and adrenal glands were reduced. These changes are shown in Table 2.

### *Effect of intravenous infusions of 2-(2-aminoethyl)pyridine*

2-(2-Aminoethyl)pyridine, 1 x $10^{-6}$ mol $kg^{-1}$ $min^{-1}$ caused a significant fall in blood pressure and total peripheral resistance. Mean blood pressure fell from 142.0 ± 2.2 mmHg before the infusion to 104.0 ± 4.0 mmHg during the infusion; mean total peripheral resistance fell from 0.31 ± 0.02 units before the infusion to 0.25 ± 0.02 units during the infusion. 2-(2-Aminoethyl)pyridine did not change cardiac output, heart rate or stroke volume significantly. These changes and the changes in tissue blood flow due to 2-(aminoethyl)pyridine are shown in Table 4. 2-(2-Aminoethyl)pyridine did not significantly increase blood flow in any tissue but significantly decreased flow to the brain, liver, spleen and skin. These tissue blood flows were associated with significant decreases in vascular resistance in the heart (41%) kidneys (43%) large intestine (47%) and small intestine (57%). 2-(2-Aminoethyl)pyridine significantly increased resistance in the spleen (29%) and skin (124%). There was no change in resistance in the brain, stomach adrenal glands, skeletal muscle or liver.

### *Effect of intravenous infusions of 4-methylhistamine*

4-Methylhistamine, 1 x $10^{-6}$ mol $kg^{-1}$ $min^{-1}$ lowered arterial blood pressure from a mean value of 110.6 ± 12.9 mmHg before the infusion to 46.4 ± 4.1 mmHg during infusion. The fall in blood pressure was due to a large significant fall in total peripheral resistance from 0.28 ± 0.03 units before the infusion to 0.13 ± 0.01 units during infusions. Heart rate, cardiac output and stroke volume were not significantly changed by 4-methylhistamine. These changes and the changes in tissue blood flow due to 4-methylhistamine are shown in Table 4.

Despite the very large fall in blood pressure during infusion of 4-methylhistamine, significant increases in blood flow occurred in the heart, stomach, and large intestine, blood flow to the small intestine was

**Table 2** The effect of histamine on blood flow and proportion of cardiac output to tissues (blood flow ml $min^{-1}$ 100 $g^{-1}$, cardiac output % of total)

| | *Histamine infusion rate mol $kg^{-1}$ $min^{-1}$* | | | | |
|---|---|---|---|---|---|
| *Tissue* | 0 | $1 \times 10^{-8}$ | $3.3 \times 10^{-8}$ | $1 \times 10^{-7}$ | $3.3 \times 10^{-7}$ |
| Brain | 63.1 ± 6.0 | 72.2 ± 5.7 | 66.5 ± 10 2 | 40 6 ± 4.6* | 31.6 ± 0.8* |
| | (7 6 ± 0.8) | (7.6 ± 1.0) | (4 7 ± 0.9)* | (5.1 ± 0 9)* | (3.0 ± 0.3)* |
| Heart | 281.3 ± 32.2 | 361.5 ± 43.0* | 593 6 ± 61.5* | 657.4 ± 53.1* | 610.3 ± 109.2* |
| | (7.9 ± 0.7) | (10.5 ± 1.3) | (12.7 ± 1.5)* | (23 1 ± 1 1)* | (17.3 ± 2.4)* |
| Kidneys | 374.1 ± 28.3 | 359 ± 42.8 | 409.1 ± 37.6* | 239.1 ± 23.3* | 220.1 ± 56.7* |
| | (21.9 ± 1.5) | (18.1 ± 1.5) | (18.3 ± 3 1) | (14.0 ± 2.1)* | (11.4 ± 2.6)* |
| Stomach | 17.3 ± 2.2 | 22.9 ± 3.2 | 40.1 ± 6.4 | 94 4 ± 18.5* | 64.5 ± 13.8* |
| | (1.2 ± 0.2) | (1 3 ± 0.1) | (1.6 ± 0 2) | (5 5 ± 1 4)* | (4.2 ± 0.6)* |
| Small intestine | 34.8 ± 4.3* | 35.3 ± 3.0 | 44 5 ± r 9 | 37 1 ± 10.5 | 56.3 ± 16.2 |
| | (9.7 ± 1.0) | (9.0 ± 0.6) | (9.2 ± 0.8) | (11.2 ± 3.0) | (15 6 ± 4 2) |
| Large intestine | 50.6 ± 8.9 | 57.6 ± 5.6 | 69 1 ± 10 7 | 54 0 ± 11 6 | 116.8 ± 20.9 |
| | (3.1 ± 0.5) | (3 8 ± 0 4) | (3.5 ± 0.5) | (4.1 ± 0.9) | (8.3 ± 2.3)* |
| Liver | 97.1 ± 8.2 | 109.8 ± 14.4 | 125.6 ± 25 7 | 54 3 ± 4.4* | 42.2 ± 8.8* |
| | (23.8 ± 2.8) | (25.6 ± 5.0) | (23.2 ± 3.2) | (14.5 ± 2.0)* | (12.3 ± 2.0)* |
| Spleen | 198 4 ± 71.4 | 130.4 ± 53.5 | 277 9 ± 94 5 | 32.9 ± 8.1* | 12.1 ± 4.3* |
| | (3.2 ± 0 9) | (2.0 ± 0.8) | (4.2 ± 1 2) | (0.6 ± 0.2)* | (0.3 ± 0.1)* |
| Adrenal glands | 469.4 ± 81.8 | 444.0 ± 62.7 | 546.8 ± 109 3 | 382 ± 54.3* | 109.3 ± 52.2* |
| | (0.7 ± 0 1) | (0.6 ± 0.1) | (0.5 ± 0 1) | (0.5 ± 0 1) | (0.3 ± 0.1) |
| Skeletal muscle | 4.4 ± 0 8 | 3.9 ± 0.8 | 3.7 ± 1 0 | 2.8 ± 0 3 | 2.0 ± 0.4 |
| Skin | 3 5 ± 0.6 | 4.8 ± 0.3 | 3.1 ± 0.7* | 1.4 ± 0.2* | 0.8 ± 0.2* |

The values in parentheses are the percentage cardiac output distributed to each tissue.
All values shown are those measured during the infusions of saline (shown as 0) or histamine. Statistical analysis of significant changes within each group was made by a paired *t* test on values before and during the infusion
* Significantly different from the pre-infusion values, $P < 0.05$

**Table 3** Effect of histamine on tissue vascular resistance (mmHg $ml^{-1}$ $min^{-1}$ 100 $g^{-1}$)

| | *Histamine infusion rate mol $kg^{-1}$ $min^{-1}$* | | | | |
|---|---|---|---|---|---|
| *Tissue* | 0 | $1 \times 10^{-8}$ | $3.3 \times 10^{-8}$ | $1 \times 10^{-7}$ | $3.3 \times 10^{-7}$ |
| Brain | 1.86 ± 0.14 | 1.68 ± 0.4 | 1.92 ± 0.36 | 1.77 ± 0.25 | 2.01 ± 0.10 |
| Heart | 0.40 ± 0.05 | 0.35 ± 0.04* | 0 19 ± 0.02* | 0.11 ± 0.01* | 0.12 ± 0.02* |
| Kidneys | 0.30 ± 0.04 | 0.41 ± 0.06 | 0.28 ± 0 03* | 0 29 ± 0.03 | 0.39 ± 0.15 |
| Stomach* | 7.33 ± 1.08 | 5.67 ± 0.76 | 3.12 ± 0.50 | 0.83 ± 0.15* | 0.98 ± 0.23* |
| Small intestine | 3.69 ± 056 | 3.53 ± 0.45 | 2.63 ± 0.30 | 2.22 ± 0.33 | 1.45 ± 0.41* |
| Large intestine | 2.77 ± 0.56 | 2.20 ± 0.34 | 1.83 ± 0.36 | 1.52 ± 0.28* | 0.59 ± 0.10* |
| Liver | 1.23 ± 0.15 | 1.17 ± 0.15 | 1.02 ± 0 14 | 1.40 ± 0 15 | 1 52 ± 0.33 |
| Spleen | 1.80 ± 0.94 | 1.39 ± 0.26 | 0.65 ± 0.20 | 3.08 ± 0 91* | 8 09 ± 2.9* |
| Adrenal glands | 0 33 ± 0.09 | 0.30 ± 0.04 | 0.33 ± 0.14 | 0.19 ± 0.03 | 0.40 ± 0.09 |
| Skeletal muscle | 34.2 ± 7.31 | 37.0 ± 5 0 | 49.9 ± 12.52 | 25.3 ± 2.26 | 34.9 ± 4.34 |
| Skin | 49.1 ± 17.61 | 27.6 ± 3.77 | 60.4 ± 23.11 | 57.0 ± 11.0 | 138.55 ± 74.7* |

All values shown are those measured during the infusions of saline (shown as 0) or histamine. Statistical analysis of significant changes within each group was made by a paired *t* test on values before and during the infusion.
* Significantly different from the pre-infusion values $P < 0.05$.

unchanged and flow to all other tissues was reduced, particularly to the liver, spleen and skin. 4-Methylhistamine caused significant decreases in resistance in the heart (by 74%), the stomach (89%), the small intestine (73%), the large intestine (77%) and skeletal muscle (49%). Resistance was not significantly changed in the brain, kidneys, liver, adrenal glands and skin but was significantly increased in the spleen (860%).

## Discussion

The depressor and vasodilator properties of histamine were first described in 1910 by Dale & Laidlaw. Although peripheral vasodilatation would inevitably contribute to the depressor responses to intravenous histamine, little is known about the possible contribution of changes in cardiac output.

Prior to our study it was reported that large doses of histamine decreased cardiac output during periods of histamine-induced circulatory collapse in dogs (Blalock & Levy, 1937). Smaller doses of histamine may cause modest and only transient increases in cardiac output (Delaunois *et al.*, 1959; Tucker *et al.*, 1975). In our experiments there was no evidence that histamine infusions, covering a dose range from $1 \times 10^{-8}$ to $3.3 \times 10^{-7}$ mol kg$^{-1}$ min$^{-1}$ had any significant effect on cardiac output. The doses used ranged from one almost without effect on blood pressure to the maximum infusion rate the cats could survive. In each case cardiac output fell slightly during the infusion but a fall of similar magnitude also occurred during infusions of saline. The sustained plateau fall in blood pressure caused by continuous intravenous infusions of histamine therefore appears to be independent of changes in cardiac output, and is due to a fall in total peripheral resistance. The measurement of cardiac output in these experiments was made after 30 min of infusion. At this time blood pressure was constant and any transient changes in cardiac output during the period before the blood pressure became constant would not have been observed.

Although the depressor response to histamine was due to peripheral vasodilatation it was clear that dilatation was not uniform in all tissues. Vasodilatation occurred in the heart at all infusion rates, the kidneys at $3.3 \times 10^{-8}$ mol kg$^{-1}$ min$^{-1}$, the large intestine and stomach at 1 and $3.3 \times 10^{-7}$ mol kg$^{-1}$ min$^{-1}$ and in the small intestine at $3.3 \times 10^{-7}$ mol kg$^{-1}$ min$^{-1}$. The dilatation was sufficient to permit increased blood flow to the heart and stomach and to maintain intestinal blood flow despite the lowered mean arterial blood pressure. Blood flow to the kidney was also increased during infusion at $3.3 \times 10^{-7}$ mol kg$^{-1}$ min$^{-1}$.

No vasodilatation occurred in the brain, liver,

**Table 4** Effect of infusions of 2-(2-aminoethyl)pyridine ($1 \times 10^{-6}$ mol kg$^{-1}$ min$^{-1}$) and 4-methyl histamine $1 \times 10^{-6}$ mol kg$^{-1}$ min$^{-1}$) on blood pressure, heart rate, cardiac output, stroke volume, total peripheral resistance and tissue blood flow

| | *2-(2-Aminoethyl)pyridine* | | *4-Methyl histamine* | |
|---|---|---|---|---|
| | *Pre-infusion* | *During infusion* | *Pre-infusion* | *During infusion* |
| Blood pressure (mmHg) | 142.0 ± 2.2 | 104.0 ± 4.0* | 110.6 ± 12.9 | 46.4 ± 4.1* |
| Heart rate (beats/min) | 247.0 ± 10.5 | 255.0 ± 24.3 | 228.0 ± 14.9 | 238.0 ± 20.0 |
| Cardiac output (ml/min) | 472.6 ± 37.5 | 436.7 ± 33.6 | 400.8 ± 50.9 | 345.8 ± 14.4* |
| Stroke volume (ml) | 1.77 ± 0.24 | 1.65 ± 0.35 | 1.77 ± 0.23 | 1.49 ± 0.13* |
| Total peripheral resistance (mmHg ml$^{-1}$ min$^{-1}$) | 0.31 ± 0.02 | 0.25 ± 0.02* | 0.28 ± 0.03 | 0.13 ± 0.01* |
| Tissue blood flow (ml min$^{-1}$ 100 g$^{-1}$) | | | | |
| Brain | 58.5 ± 4.4 | 43.5 ± 5.9* | 69.1 ± 9.4 | 27.4 ± 4.9* |
| Heart | 420.7 ± 53.9 | 517.4 ± 86.2 | 266.0 ± 46.0 | 404.4 ± 46.0* |
| Kidneys | 346.5 ± 31.9 | 452.9 ± 32.4 | 357.1 ± 37.0 | 168.2 ± 24.0* |
| Stomach | 36.3 ± 5.1 | 25.1 ± 1.5 | 38.1 ± 16.1 | 94.2 ± 8.4* |
| Small intestine | 55.4 ± 9.1 | 58.3 ± 7.9 | 49.4 ± 11.4 | 67.6 ± 10.2 |
| Large intestine | 90.7 ± 20.4 | 112.9 ± 18.4 | 81.5 ± 14.3 | 138.4 ± 7.2* |
| Liver | 137.8 ± 43.8 | 97.2 ± 25.8* | 67.5 ± 6.9 | 19.9 ± 4.4* |
| Spleen | 324.3 ± 48.1 | 78.1 ± 21.1* | 217.4 ± 31.7 | 26.1 ± 9.4* |
| Adrenal glands | 678.6 ± 231.8 | 630.7 ± 145.6 | 525.1 ± 130.7 | 215.6 ± 85.2* |
| Skeletal muscle | 3.8 ± 0.6 | 3.7 ± 0.7 | 3.8 ± 0.9 | 2.6 ± 0.5 |
| Skin | 4.4 ± 0.9 | 1.5 ± 0.3* | 5.8 ± 1.9 | 1.6 ± 0.2* |

* Significantly different from the pre-infusion values $P < 0.05$.

adrenal glands or skeletal muscle at any dose level and vasoconstriction occurred in the spleen and skin at the two higher histamine infusion rates.

Histamine-induced vasodilatation in the heart has been described previously, usually in *in vitro* preparations (see review by Rocha e Silva, 1966), although Parratt (1969) also reported that intravenous injections of histamine increased coronary blood flow in anaesthetized monkeys. In our experiments coronary vasodilatation was dose-dependent and could be detected during all infusions of histamine. Coronary vasodilatation was the only significant histamine-induced change detected at the lowest histamine infusion rate. Histamine infusions lowered blood pressure with no change in cardiac output, and so decreased cardiac work. It seems likely that the coronary vasodilatation caused by histamine was due to a direct effect on the coronary circulation rather than a metabolic response to increased cardiac work.

Histamine-induced increases in gastric blood flow, usually during periods of histamine-induced acid secretion, are well documented (e.g. Harper, Reed & Smy, 1968; Jacobsen & Chang, 1969). The doses of histamine used in this study are known to stimulate acid secretion in cats (Harper *et al.*, 1968; Parsons, personal communication) and it is reasonable to assume that acid secretion was increased during the larger infusions used in these experiments. The histamine-induced vasodilatation might, therefore, have been due to a direct effect on the gastric vasculature or metabolic response to the increased gastric work during acid secretion.

In the kidneys, histamine appeared to cause some vasodilatation when blood pressure was only slightly reduced. The dilatation was less at higher infusion rates, and renal blood flow decreased. The kidneys play a major role in the reflex control of systemic blood pressure and many vasodilator compounds cause an increased secretion of renin from the kidney (e.g. Pettinger, Campbell & Keeton, 1973). Renin release may well occur during the sustained hypotensive response to histamine. The renal vascular response to histamine might reflect a complex balance between histamine-induced vasodilatation and renin/angiotensin-induced vasoconstriction.

Histamine dilates intestinal resistance vessels after intra-arterial administration (Lee, 1957; Flynn & Owen, 1975). In these experiments vasodilatation could be detected during intravenous infusions of histamine. The dilatation was less marked than in the heart or stomach but adequate to maintain flow during histamine-induced hypotension. Similar results were recently reported by Krarup (1975) using flow probes to measure intestinal blood flow.

Histamine had no effect on vascular resistance in the brain, liver, adrenal glands and skeletal muscle, and blood flow fell as blood pressure fell. Although there were no significant changes in hepatic vascular resistance, there was a tendency for resistance to increase during the larger infusions of histamine. Krarup (1975) recently reported significant increases in hepatic vascular resistance during intravenous infusions of histamine.

Histamine increased vascular resistance in the spleen and skin. These increases occurred during infusions of histamine which lowered mean arterial blood pressure. Sympathetic stimulation is particularly effective in causing vasoconstriction in these organs. The increase in vascular resistance in the spleen and skin is probably part of the homeostatic reflex mechanisms initiated by the fall in mean blood pressure.

Both 2-(2-aminoethyl)pyridine, a histamine $H_1$-receptor agonist (Durant *et al.*, 1975) and 4-methylhistamine, a histamine $H_2$-receptor agonist (Black *et al.*, 1972) lowered blood pressure and decreased total peripheral resistance. Both compounds tended to reduce cardiac output but the change was similar to the change during saline infusions. The depressor response seems therefore to be due entirely to peripheral vasodilatation. 2-(2-Aminoethyl)pyridine did not significantly increase blood flow to any tissue although vascular resistance was decreased in the heart, kidneys and small and large intestines but increased in the spleen and skin. 4-Methylhistamine reduced vascular resistance in the heart, stomach and small and large intestines, and increased resistance in the spleen and skin. The coronary and intestinal vasodilatation during histamine infusions was qualitatively mimicked by both histamine receptor agonists whereas vasodilatation in the stomach was mimicked only by 4-methylhistamine.

These results suggest that both histamine $H_1$- and $H_2$-receptors are present in the coronary and intestinal vasculature and both can mediate vasodilatation. In contrast, vasodilatation in the stomach, whether a direct vascular effect or a metabolic response to acid secretion, appears to be an $H_2$-receptor phenomenon.

Vasoconstriction in the spleen and skin was caused by both agonists and is probably part of the reflex response to the hypotension caused by each agonist.

2-(2-Aminoethyl)pyridine caused renal vasodilatation whereas 4-methylhistamine did not. The renal vascular response to histamine was complex and was probably due to a balance between the direct vasodilator effects of histamine and indirect reflex effects associated with the hypotension. Meaningful comparison of the two agonists would therefore require similar falls in blood pressure. The apparent different effects of the agonists on renal vasculature may not reflect different direct effects on the vascular bed.

In conclusion, it has been shown that the sustained fall in blood pressure during infusion of histamine is due to peripheral vasodilatation. This occurs predominantly in the heart, stomach and intestine; there is no vasodilatation in the brain, liver, adrenal glands or skeletal muscle. The selective histamine receptor agonists, 2-(2-aminoethyl)pyridine and 4-methylhistamine also lower blood pressure by causing peripheral vasodilatation.

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# THE EFFECT OF SULPHAMETHOXYPYRIDAZINE ON LIVER AND PLASMA LEVELS OF VITAMIN A IN RATS

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1 Long-acting sulphonamides are highly bound to plasma proteins; the present study deals with the effects of this binding property upon a vitamin A compound also transported by plasma proteins.

2 Sulphamethoxypyridazine was administered in rats either orally or by intraperitoneal injection.

3 A significant fall in plasma vitamin A level and an increase in hepatic vitamin A concentration were observed.

4 These results suggest an interference by sulphamethoxypyridazine with the transport of vitamin A, either through competition between the drug and vitamin A for binding sites of plasma proteins, or through altered secretion of the vitamin from the liver.

## Introduction

During the past 15 years there has been increasing awareness that many commonly prescribed drugs can interact with endogenous compounds for binding sites in cells and plasma proteins (Anton, 1973), such interaction possibly affecting the concentration of the circulating endogenous compounds. These studies have been carried out especially on hormone transport (Oppenheimer, 1968; 1973; Larsen, 1972), but few data have been reported about interactions with plasma-protein transported vitamins. It is also widely recognized that most, if not all drugs are bound to plasma proteins for transport, but the role of this phenomenon in determining their overall pharmacological properties has not yet been clearly defined. During the past decade, a type of long-acting sulphonamide with high affinity for plasma proteins has been described (Weinstein, 1965), one of these, sulphamethoxypyridazine (SMP) being bound to plasma albumin to the extent of 85%. Since vitamin A is also transported by plasma proteins forming a complex with Retinol Binding Protein (RBP) α-globulin (Goodman, 1973) it seemed of interest to study the interaction between vitamin A and sulphonamides.

This paper describes the effect of high doses of SMP on plasma and hepatic vitamin A levels in rats.

## Methods

Adult Fischer rats of either sex (CDF Fischer 344) bred in this department were used throughout all the experiments. The animals were housed individually and maintained on a standard commercial rat chow (containing 24% protein) and tap water *ad libitum* in a room where lighting and temperature (23 ± 2°C) were controlled.

### *Experiment I: Plasma vitamin A levels after sulphamethoxypyridazine administration*

Two separate groups of 10 animals were given a buffered saline solution (pH 8) of SMP (14 mg/100 g body weight) by stomach tube once daily; they were killed after 7 and 14 days respectively. The control group (10 animals) received an equal volume of saline (0.9% w/v NaCl solution). The body weight was recorded daily and the skin, eyes and oral mucosa were observed carefully. Routinely, animals were starved overnight before they were killed. Animals were exsanguinated and blood was collected. Plasma vitamin A levels were measured according to the method of Bessey, Lowry, Brock & López (1946).

### *Experiment II: Relationship between dose of sulphamethoxypyridazine administered and plasma vitamin A levels*

(a) *Intragastric administration.* Thirty-five three-week old rats of similar weight (50 ± 3 g) were distributed randomly into 5 groups receiving SMP in the following daily doses by stomach tube for 20 days: 1.4; 2.8; 4.2 and 7.1 mg/100 g body weight, the control group receiving saline. The animals were observed for overt signs of toxicity and body weights were recorded daily. The rats were killed by exsanguination 16 h after the last administration of SMP. SMP and vitamin A were determined in plasma by the method of Bratton & Marshall (1939), and Bessey *et al.* (1946) respectively; the liver was homogenized in distilled water at 0.1% and vitamin A was determined in the homogenates.

(b) *Intraperitoneal administration:* Fifty three-weeks old rats were distributed in five groups receiving SMP in buffered (pH 6.9) saline in the doses indicated above. All determinations were run in triplicate. In all cases the significance of differences between results

**Table 1** Effect of sulphamethoxypyridazine (SMP) treatment on vitamin A plasma levels

| *Length of treatment with SMP* | *Plasma vitamin A level (µg/100 ml)* |
|---|---|
| No treatment (controls) | 35 3 ± 5 02 (10) |
| 7 days | ND (10) |
| 14 days | ND (10) |

ND = not detectable.
Animals were treated as described in the Methods section under Experiment I. The values represent the means ± s e. mean with the number of observations in parentheses.

**Table 2** Effect of different doses of sulphamethoxypyridazine (SMP) administered by stomach tube on vitamin plasma level

| *Groups* | *No. of animals* | *Age of animal in days*[1] | *SMP (µg/100 ml)* | | *Vitamin A (µg/100 ml)* |
|---|---|---|---|---|---|
| Control | 7 | 42 | | | 34.57 ± 4 04 |
| *SMP treated* | | | | | |
| 1 4 mg/100 g body weight | 7 | 42 | [2]FS | = 0 045 | 24.59 ± 5.22* |
| | | | [3]TS | = 0 062 | |
| 2.8 mg/100 g body weight | 7 | 42 | FS | = 0 057 | 25.53 ± 4 60** |
| | | | TS | = 0.085 | |
| 4.2 mg/100 g body weight | 7 | 42 | FS | = 0 071 | 18 35 ± 4.64** |
| | | | TS | = 0 114 | |
| 7.1 mg/100 g body weight | 7 | 42 | FS | = 0.423 | 1.27 ± 1 20 |

[1] Age at the time animals were killed; [2] FS free SMP; [3] TS total SMP.
Animals were treated as described in the Methods section under Experiment IIa. The values represent the means ± s.d. of 7 observations.
* $P<0.02$; ** $P<0.01$; *** $P<0.001$, when compared with corresponding control values

**Table 3** Effect of different doses of sulphamethoxypyridazine (SMP) administered intraperitoneally on SMP in plasma and on hepatic vitamin A levels

| | | | *Plasma* | | | *Liver* |
|---|---|---|---|---|---|---|
| *Groups* | *No. of animals* | *Age of animals in days*[1] | *Sulphonamide (µg/100 ml)* | | *Vitamin A (µg/100 ml)* | *Vitamin A (µg/g)* |
| Control | 10 | 42 | | | 33 76 ± 6.19 | 104.79 ± 25 87 |
| *SMP treated* | | | | | | |
| 1 4 mg/100 g body weight | 10 | 42 | [2]FS | = 0 043 | 23.91 ± 4.48* | 80 06 ± 22 56 |
| | | | [3]TS | = 0.062 | | |
| 2 8 mg/100 g body weight | 10 | 42 | FS | = 0 047 | 14 52 ± 6.01** | 193.55 ± 23.41 |
| | | | TS | = 0 095 | | |
| 4.2 mg/100 g body weight | 10 | 42 | FS | = 0 069 | 14 14 ± 2.12** | 164.84 ± 16.00** |
| | | | TS | = 0.185 | | |
| 7.1 mg/100 g body weight | 10 | 42 | FS | = 0 100 | 4.84 ± 2.93** | 200.48 ± 16 29** |
| | | | TS | = 0.127 | | |

[1] Age at the time animals were killed; [2] FS free SMP; [3] TS total SMP.
Animals were treated as described in the Methods section under Experiment IIb The values represent the mean ± s.d. of 10 observations.
* $P<0.01$; ** $P<0.001$, when compared with corresponding control values

was analysed by the use of Student's test for independent means (Snedecor, 1956).

## Results

### *Effect of sulphamethoxypyridazine on plasma vitamin A levels*

Table 1 shows that the intragastric administration of high doses of SMP caused a drop in plasma vitamin A circulating levels which was already evident after 7 days treatment. Clinically the animals showed skin and mucosal lesions even at the fifth day of treatment: 7 of 10 animals receiving SMP for 14 days showed xerophthalmia, nasal chaps, red conjuntiva with scabs in the eyelids.

### *Dose-effect relationship*

The stomach tube administration of SMP over 20 days caused a retardation of body weight gain, which correlated well with the dose. Similar findings were obtained with intraperitoneal administration of SMP over the same period of time.

Table 2 shows the effect of different doses of SMP on plasma vitamin A levels. A relationship was observed between the dose of SMP administered and the plasma vitamin A level, so that the higher the dose, the greater the drop in plasma vitamin A concentration.

When the sulphonamide was administered intraperitoneally, a drop in plasma vitamin A levels was also observed and, at the same time, a significant increase in hepatic vitamin A concentration was found (Table 3).

## Discussion

Our results have demonstrated that prolonged administration of high doses of SMP either by stomach tube or by intraperitoneal injection causes a fall in vitamin A plasma levels which is already noticeable (results not shown) on the fifth day of administration. These low levels of vitamin A in plasma are accompanied by the typical skin and mucosal lesions of avitaminosis A.

Treated animals also show an increase in hepatic vitamin A level. It is known that under physiological conditions vitamin A is stored in liver and transported by the plasma (Garbers, Gillman & Peisach, 1960) after its binding to a complex formed by the prealbumin and $\alpha$-globulin (Goodman, 1973). SMP is also transported by the plasma largely bound to plasma proteins (Anton, 1973), mainly to albumin and when its blood concentration is very high, also to globulins. It is therefore possible to explain the present results on the basis of a competition between vitamin A and SMP for binding sites of plasma proteins, although it is also possible that SMP interferes in some other way with the secretion of vitamin A from the liver into the blood.

The doses of SMP administered to the rat in these experiments are high as compared with those used therapeutically in man. Nevertheless, the present results raise the possibility that patients given the drug for a long time may develop alterations in vitamin A metabolism.

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# AN EXCITATORY ACTION OF IONTOPHORETICALLY ADMINISTERED LITHIUM ON MAMMALIAN CENTRAL NEURONES

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1 The action of iontophoretically administered lithium was studied on spinal Renshaw cells and interneurones and on supraspinal neurones in cerebral cortex, thalamus, hypothalamus and brain stem in anaesthetized cats and rats.

2 There was a correlation between the effects of $Li^+$ and those of acetylcholine (ACh), although rather more cells were unaffected by $Li^+$ than by ACh.

3 The usual effect was an excitation of rather slow onset, but occasionally effects were produced with time courses similar to those of ACh. The excitation was blocked by ACh antagonists and was best demonstrated with dihydro-β-erythroidine on Renshaw cells.

4 The postsynaptic excitant action of ACh on Renshaw cells was reduced by $Li^+$.

5 Depressant actions of $Li^+$ were encountered on cells also depressed by ACh.

6 It is concluded that $Li^+$ may facilitate cholinergic transmission at some sites in the CNS by increasing the release of ACh by an unknown mechanism. Similar effects at non-cholinergic synapses might also occur but would appear to be of less importance. Since facilitation of neuronal firing with $Li^+$ was usually observed, the depressant effects on postsynaptic responses to ACh may be of little consequence.

## Introduction

In recent years, lithium salts have come into prominence in the management of affective disorders, especially in the treatment of manic-depressive psychosis. Despite many investigations, the mechanism by which lithium exerts its therapeutic effects remains obscure (for recent reviews and references see Gershon & Shopsin, 1973; Johnson, 1975; Bunney & Murphy, 1976). One difficulty is that therapeutic effects are obtained at low plasma levels of 0.8–1.5 mequiv/l and only after repeated administration for at least several days (Coppen, 1973). The delay in onset of the therapeutic effect may be due to the fact that this plasma level, which is the maximum that can be tolerated without untoward side-effect, cannot rapidly build up an effective concentration at a central site of action. This difficulty might be overcome by administration of lithium directly at its site of action on central neurones. In this respect, the iontophoretic method used in this study represents the best technique currently available, but suffers from the disadvantage that it may only examine acute, rather than long term effects (Segal, 1974). However, if the therapeutic delay is only due to the slow kinetics of accumulation of lithium at central sites, then this disadvantage of the electrophoretic technique may not be important.

In the majority of *in vitro* studies rather high concentrations of lithium have been employed and these may be expected to produce nonspecific actions on excitable membranes by virtue of the fact that lithium substitutes for sodium in diffusion across cell membranes but is not handled effectively by the sodium pump. In one exceptional study with low concentrations of 1–5 mM (Ploeger & den Hertog, 1973), it was shown that lithium affected the activity of the sodium-potassium pump in isolated nerve fibres, probably by competition between lithium and potassium ions.

Such effects of lithium on ionic mechanisms are unlikely to affect central neurones in a specific fashion unless some aspects of the transmission process, or particular central neurotransmitters are more dependent upon a particular ionic process than others. Thus, it is not impossible to reconcile such a general basic mechanism of action with a modification of central neurotransmission specific enough to explain the rather selective therapeutic action of lithium and there is a great deal of mainly biochemical evidence showing changes in the levels, turnover or release of certain putative transmitters.

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Our interest in a possible link between the therapeutic action of lithium and cholinergic mechanisms arose from observations by Lee, Lingsch, Lyle & Martin (1974) and Lingsch & Martin (1976) that choline uptake into human erythrocytes is greatly reduced in lithium-treated patients and that the effect seemed to be irreversible and independent of the presence of lithium at the time of measurement. It seemed possible that a similar process occurring in central cholinergic terminals might reduce acetylcholine (ACh) synthesis, thereby impairing cholinergic transmission.

Although this working hypothesis was not substantiated by the present investigation, evidence was obtained which indicated that lithium probably does modify central cholinergic transmission through a presynaptic action, although the effect is probably not absolutely specific for cholinergic terminals.

A preliminary report of this study was presented to the British Pharmacological Society (Haas & Ryall, 1974).

## Methods

This study was carried out on 207 neurones in 40 cats and 21 neurones in 7 rats. Cats were anaesthetized either with pentobarbitone sodium (35 mg/kg i.p.) or with chloralose (60 mg/kg i.v.) after halothane induction and anaesthesia was supplemented when required by intravenous injection. A few experiments were performed under continuous halothane anaesthesia. Rats were anaesthetized with a mixture of urethane and pentobarbitone (400 mg/kg plus 5 mg/kg i.p.) supplemented by pentobarbitone when required.

In experiments on spinal neurones, the cord was exposed by a laminectomy extending from L2 to L7 and ventral roots S1 and L7 were dissected free and mounted upon bipolar electrodes for stimulation in order to identify Renshaw cells by their characteristic discharge to antidromic volleys in ventral roots. Interneurones were located by their response to stimulation of a dorsal root or a peripheral nerve in the ipsilateral hind limb; they were not excited by antidromic volleys in ventral roots.

The head of the animal was fixed in a stereotaxic frame for location of supraspinal neurones. Cortical neurones were located in the pericruciate region but were not further identified. Thalamic neurones were located by stereotaxic coordinates and some were identified as presumed relay neurones if they were excited at short latencies on stimulation of the median nerve or medial lemniscus. A few were also identified by antidromic invasion from the cerebral cortex. Hypothalamic neurones were approached stereotactically from the dorsal side (cats) or transpharyngeally in rats (Dreifuss & Kelly, 1972) and some were identified as neurosecretory neurones by antidromic invasion from the hypophyseal stalk.

Extracellular records were obtained via the centre barrel of multi-barrelled micropipettes with overall tip diameters of 5–8 μm or via a projecting barrel of a parallel micropipette assembly. Recording barrels were filled with 4 M NaCl. After suitable amplification and discrimination of single units, standard pulses were led into an R–C ratemeter or a digital counter. The input to the counter could be gated so that action potentials evoked in response to nerve stimulation could be counted for a variable time after the stimulus, thus avoiding counting background 'spontaneous' firing of the cell. The counter was reset only after many (10–50) stimulus presentations. The chart record therefore continuously displayed either mean firing frequency or average number of discharges after a stimulus.

Exposed spinal cord was covered by a pool of warmed paraffin oil and the animal was maintained at about 37–38°C by heating pads automatically controlled by a thermistor-operated circuit.

Outer drug containing barrels of the micropipettes contained LiCl (1 M), ACh bromide (0.5 M), atropine sulphate (0.01 M in 0.16 M NaCl), dihydro-β-erythroidine (DHBE, 0.02 M in 0.16 M NaCl), NaCl (4 M) or, occasionally, L-glutamic or DL-homocysteic acid (Na salt, 0.2 M, pH 8). Retaining voltages of 0.5 V were routinely applied to drug-containing barrels. Typical electrode resistances ranged from 5–10 MΩ for recording barrels and 20–100 MΩ for drug-containing barrels.

## Results

### *The effect of lithium on Renshaw cells*

Lithium was administered with electrophoretic currents up to $400 \times 10^{-9}$ A to 39 Renshaw cells in 10 cats, while continuously observing discharges to submaximal (18 cells) or maximal (2 cells) ventral root stimulation, or background, 'spontaneous' firing (20 cells) or responses to periodic iontophoretic administration of ACh (10 cells). Submaximal stimulation of ventral roots produced rather variable discharges and these were counted and automatically averaged for a number of repetitions and continuously plotted on a pen recorder; submaximal rather than maximal stimulation was employed initially in anticipation of an expected decrease in the response but was continued in later experiments when it became apparent that the effects of $Li^+$ were more likely to be excitatory than depressant. On some cells combinations of these techniques were employed.

Excitatory effects of $Li^+$ were encountered in 24 of 39 cells examined. The characteristics of the change in excitability varied from cell to cell and even sometimes changed on repeated administration to the same cell. This variability in the response presented a major difficulty in analysing the mechanism of the excitatory

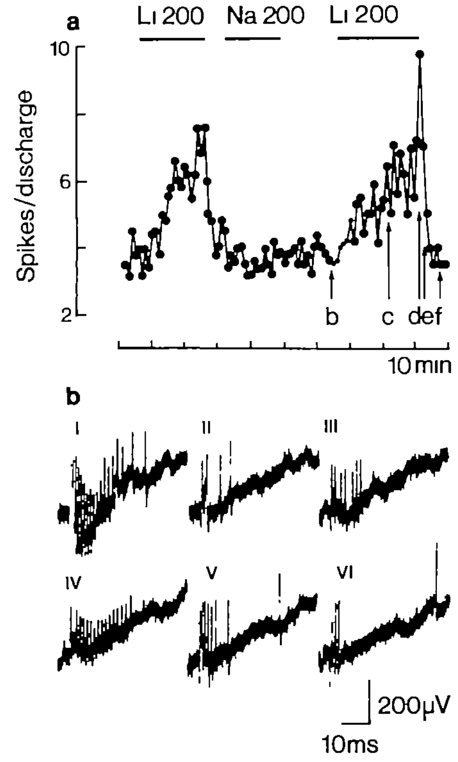

**Figure 1** (a) Effect of iontophoresis of Li$^+$ ($200 \times 10^{-9}$ A) or Na$^+$ on the submaximal discharge of a Renshaw cell evoked by ventral root stimulation Each point is the average of 10 discharges Ordinate scale: average number of spikes per discharge. Abscissa scale; intervals of 10 minutes. (b) A sample photograph of a maximal discharge in (i) and sample records of submaximal discharges before (ii), during (iii, iv) and after (v, vi) Li$^+$ iontophoresis at the times indicated in (a). Calibrations 200 µV and 10 milliseconds. Photographs taken on film moving along the vertical axis.

effect, particularly when using antagonists, and successful experiments with antagonists were only possible on relatively few occasions.

The most usual effect encountered was a very slow increase in either the average rate of background discharge (in 10 of 20 cells tested) or in the number of action potentials evoked by a submaximal ventral root volley (in 13 of 18 cells tested). The electrophoretic currents employed usually ranged from 100 to $300 \times 10^{-9}$ A. The evaluation of smaller currents was usually precluded by the variability in the response with repeated applications but on the few occasions when they were tested they were less effective.

One of the earliest signs that Li$^+$ would ultimately produce an effect was often that action potentials began to occur in bursts. As the excitatory effect progressed so the repetition rate of the bursts and the frequency within the bursts tended to increase.

Figure 1 illustrates a typical effect of $200 \times 10^{-9}$ A Li$^+$ on the discharge of a Renshaw cell evoked by submaximal stimulation of L7 ventral root. Each point in Figure 1a is the average of 10 repeated stimuli. In the absence of Li$^+$ there was an average of about four spikes in each discharge, compared with 14 spikes evoked by maximal antidromic volleys (Figure 1b(i)). During the administration of Li$^+$ the magnitude of the evoked discharge increased slowly over a period of about 20 min and returned to control values when Li$^+$ was discontinued. The effect on this cell was reproduced by a second administration. The effects were not considered to be due to the currents themselves for several reasons. The effects were not reproduced by the administration of Na$^+$ ions with the same electrophoretic current, nor were they counteracted by the simultaneous administration of a 'balancing' current ejecting Cl$^-$ from another barrel (not illustrated). Finally, the slow onset of the effect renders it unlikely that the excitation was due to the current *per se*.

A reduction in spike amplitude was often seen during the high frequency firing evoked by Li$^+$ and in the discharges evoked trans-synaptically during intense excitation by Li$^+$ (Figure 1b(iv)). No particular significance can be attached to this observation because Renshaw cells tend to show spike inactivation during high frequency firing (see control record in Figure 1b(i)), regardless of the method used to evoke such firing.

An effect similar to that in Figure 1 is also shown in Figure 2a on another cell. On this cell (Figure 2b, c) the effect on background activity is also illustrated. It may be seen that, during and after Li$^+$ administration, the variability in the background activity was increased due to the occurrence of 'burst' activity (Figure 2b).

Reproducible excitatory effects on background activity were seen on only a few occasions. An example is shown in Figure 4f in which Li$^+$ produced an excitation similar to that produced by ACh. On other cells such an excitation was observed only during the first administration of Li$^+$ and was either much reduced or absent subsequently. Transient 'burst-like' firing was seen on 3 cells, but on one of these it was only apparent a short while after the administration of ACh (Figure 3c) for a few administrations of Li$^+$. An extreme example of 'burst-like' firing is shown in Figure 4a, b. On some cells the increase in burst firing did not recover within the period of observation after cessation of Li$^+$ administration.

Thus Li$^+$ caused an increase in synaptically evoked discharges and background activity with a variable time-course and reproducibility which did not seem to be due to current artefacts. Such changes could have

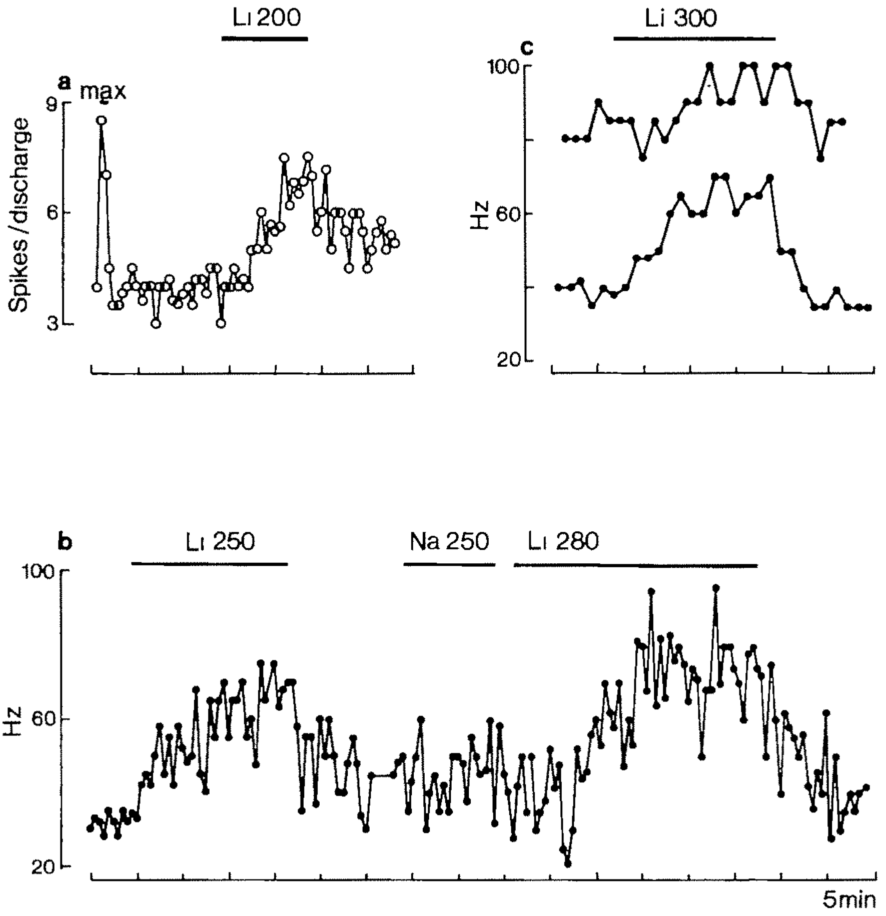

**Figure 2** Effects of $Li^+$ iontophoresis on a Renshaw cell. The average of 10 discharges to a brief period of maximal stimulation (max) of the ventral root and, subsequently, repeated submaximal activation at 10 Hz is shown in (a), (b) and (c) show the action of $Li^+$ and $Na^+$ on background activity (b and lower record in c) and on the maximum frequency of firing attained during the repeated iontophoresis of acetylcholine (ACh) with a current of $8 \times 10^{-8}$ A (upper record in c). Despite the slight increase in maximal firing frequency to ACh (c) the elevation in firing frequency above the background rate (the difference between the upper and lower records in c) was reduced. Ordinate scales; average number of action potentials per discharge in (a), frequency of firing (Hz) in (b) and (c). Abscissa scale; 5 min intervals.

been produced by either pre- or postsynaptic effects. The effects of $Li^+$ on the firing evoked by ACh was therefore tested on 10 cells on the assumption that ACh causes firing by an action on postsynaptic receptors.

On 2 cells there was no change in the response to ACh. On another (Figure 3a) there was an increase in the background discharge and a slight reduction in the increase in frequency caused by ACh although there was a slight elevation in the maximum firing frequency. On the remaining cells, the effect of ACh was reduced, even in the absence of changes in background activity (Figure 3b).

It therefore seemed possible that $Li^+$ might excite Renshaw cells by causing a release of transmitter from presynaptic terminals. The only identified excitatory transmitter on Renshaw cells is ACh and dihydro-β-erythroidine (DHBE) is an ACh antagonist at the nicotinic receptors (Curtis & Ryall, 1966a,b). DHBE was therefore tested by iontophoresis on 4 cells on which the excitatory effect of $Li^+$ was either repeatable or maintained during the administration. On all 4 cells the excitatory effect of both ACh and $Li^+$ was blocked by DHBE (Figure 4b,f). Some recovery of the $Li^+$ effect is evident in Figure 4f but the cell in (b) was lost before recovery from DHBE was observed.

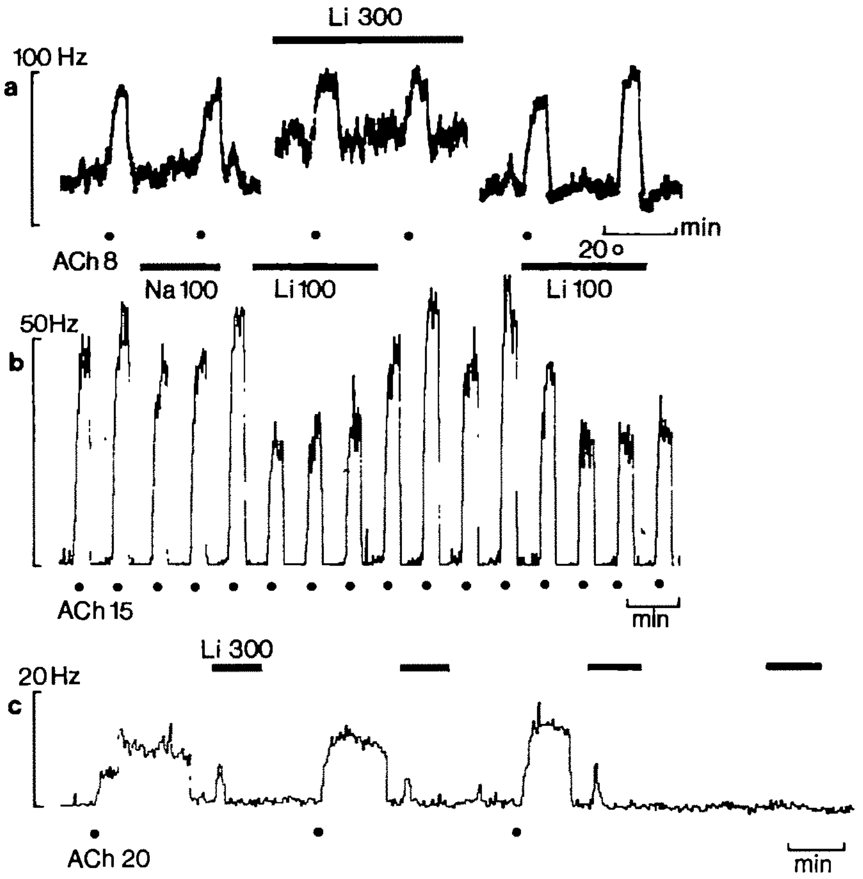

**Figure 3** Interaction between Li$^+$ and acetylcholine (ACh) on Renshaw cells. Data obtained on 3 different cells (a), Li$^+$, $300 \times 10^{-9}$ A elevated background firing rate with only a small reduction in the increased firing frequency produced by ACh, $8 \times 10^{-9}$ A. (b) Li$^+$, $100 \times 10^{-9}$ A decreased ACh-evoked responses with no increase in background activity: Na$^+$, $100 \times 10^{-9}$ A had no clear effect. (c) Li$^+$, $300 \times 10^{-9}$ A caused transient bursts of firing only when administered a short time after the administration of ACh Calibrations; firing frequency in Hz and minutes. Administration of Li$^+$ is indicated by the horizontal bars above the records. ACh (●) was administered for periods of 20 s, (a, b) or 60–100 s (c).

### *The effect of hemicholinium on Renshaw cells*

The original rationale for the lithium experiments was based upon a possible hemicholinium (HC-3)-like action in blocking the uptake of choline (see Introduction). HC-3 was therefore tested upon 13 Renshaw cells. On 4 cells diffusion of HC-3 from the micropipette, achieved by removing the retaining current, caused a gradual decline in the number of spikes elicited in each discharge to ventral root stimulation and it was observed that even in the presence of the retaining current on 2 cells there was a gradual decline in the evoked response after first locating the neurone. On 1 cell currents of 15–70 nA caused no apparent change in the evoked response. On the remaining 8 cells hemicholinium caused a mixture of depressant and excitatory effects. The depression was seen usually as an increased latency of the first spike in the evoked response (Figure 5b–d), sometimes accompanied by a small decrease in the maximal evoked response. The excitation was revealed as a marked increase in the number of discharges in an evoked submaximal response (Figure 5b–e). The excitatory effect was usually the most obvious action of HC-3 and tended to be fast in onset, although on one cell a slow time course was observed, similar to that observed with Li$^+$.

The increased latency to the first action potential after HC-3 administration in the presence of facilitation of submaximal discharges is difficult, if not

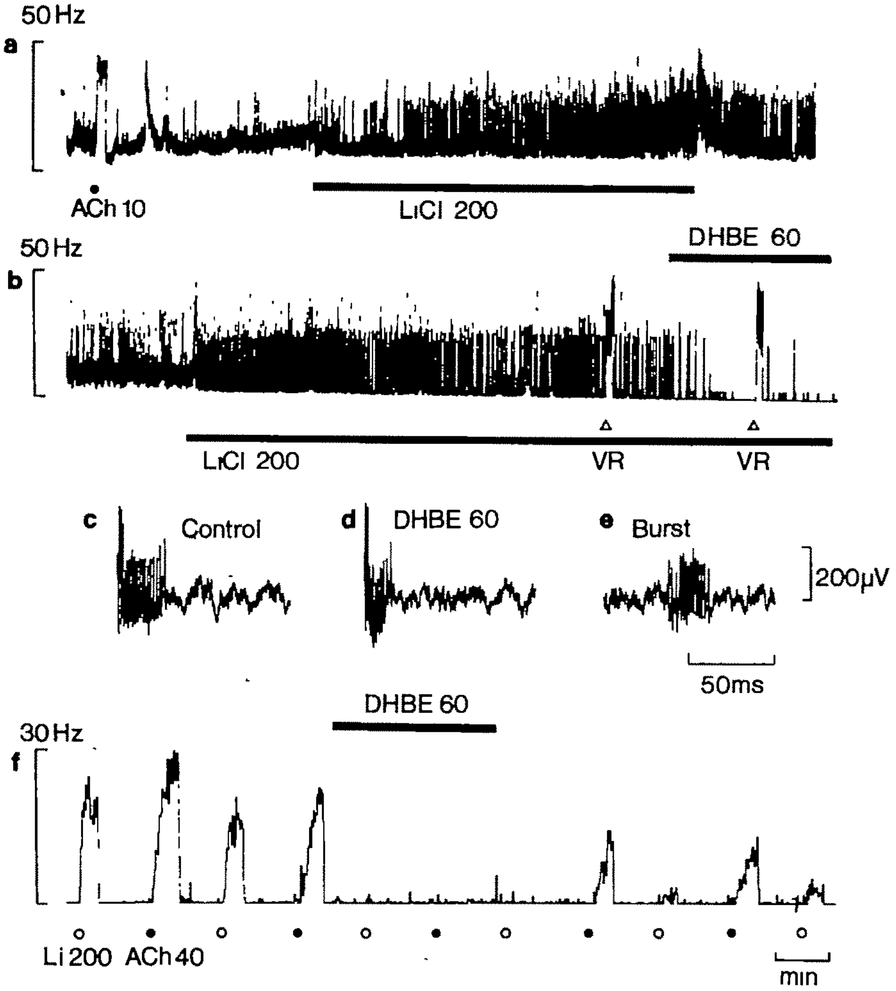

**Figure 4** Interaction between excitation by $Li^+$ and dihydro-β-erythroidine (DHBE) on two Renshaw cells (a–e and f). The cell in (a) was excited by acetylcholine (ACh) $10 \times 10^{-9}$ A. $Li^+$, $200 \times 10^{-9}$, compensated by the simultaneous ejection of $Cl^-$ from another barrel, caused the appearance of 'burst'-like firing which increased gradually and only slowly waned after the cessation of $Li^+$ administration. A typical record of a single 'burst' is shown in (e). The effect was repeatable with a second administration of $Li^+$ in (b) and the 'bursts' were abolished by the administration of DHBE ($60 \times 10^{-9}$ A), which reduced the maximal discharge to antidromic ventral root (VR) stimulation (c and d). (f) Firing produced by $Li^+$ (O) on this cell was similar to that produced by ACh (●) and was unusually fast in onset and repeatable. DHBE ($60 \times 10^{-9}$ A) abolished responses to both ACh and $Li^+$, with partial recovery after the termination of the DHBE ejecting current. Calibrations: firing frequency in Hz and min in (a), (b) and (f); 200 μV and 50 ms in (c–e). Duration of $Li^+$ administration indicated by horizontal bars below records in (a) and (b) and of DHBE administration by bars above the records in (b) and (f). Four min interval between records (a) and (b)

impossible to interpret because the former probably represents a presynaptic and the latter a postsynaptic action. The postsynaptic nature of the facilitation is indicated, but not proved, by the excitation of non-cholinoceptive interneurones.

In general, the effects of HC-3 on Renshaw cells confirm the observations by Quastel & Curtis (1965).

### *Comparison of actions of $Li^+$ and hemicholinium on interneurones*

Lithium, administered with electrophoretic currents of $300$–$400 \times 10^{-9}$ A failed to excite any of the 6 spinal interneurones on which it was tested. ACh was also ineffective on these cells. In contrast, HC-3

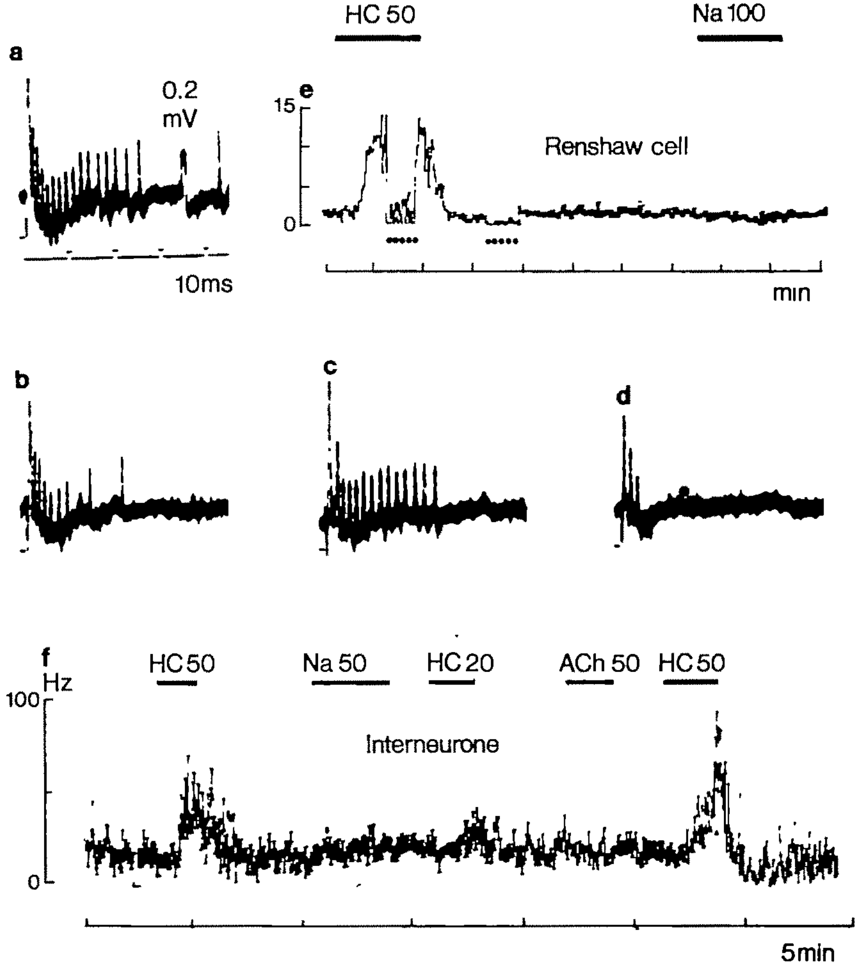

**Figure 5** The action of hemicholinium (HC-3) on a Renshaw cell (a–e) and an interneurone (f). Records of a synaptically evoked discharge evoked by maximal (a) and submaximal stimulation of the ventral root before (b), during (c) and after (d) the iontophoretic administration of HC-3 with $50 \times 10^{-9}$ A. Calibrations for (a–d) are shown in (a). The trace in (e) shows the average number of spikes in each submaximal discharge to a ventral root volley. During the period indicated by .. ... the rate of stimulation was reduced from 5 Hz to 1 Hz, resulting in fewer counts in each counting epoch; records (c) and (d) were obtained at these times. A control current of $100 \times 10^{-9}$ A ejecting $Na^+$ was ineffective. Time intervals on the abscissa scale are in minutes. HC-3 ($20–50 \times 10^{-9}$ A) also increased the 'spontaneous' background discharge of an interneurone (f) which was insensitive to ACh ($50 \times 10^{-9}$ A) and $Na^+$ ($50 \times 10^{-9}$ A). Ordinate scale: average firing frequency in Hz. Abscissa scale: time intervals are 5 minutes.

(20–100 nA) excited all 4 interneurones on which it was tested (Figure 5f). Thus the excitatory action of $Li^+$ appeared to be selective for Renshaw cells, whereas the excitation by HC-3 was not.

### *The effect of lithium on supraspinal neurones*

Table 1 shows an analysis of the data obtained on 157 supraspinal neurones in 21 cats and 7 rats.

A total of 123 cells was excited by ACh, 9 were inhibited and 25 were unaffected. Lithium excited 85 neurones, inhibited 7 and had no effect on the remainder.

There was a close correlation between the effect of ACh and that of $Li^+$. Lithium and acetylcholine had similar effects on 91 cells, and inhibition by $Li^+$ was only observed on thalamic neurones, which were the only cells on which depression by ACh was noted. On

**Table 1** A comparison of the action of $Li^+$ and acetylcholine (ACh) on 157 supraspinal neurones in cats and rats

| *Site* | *Effect of ACh* | *Number of cells affected by $Li^+$* | | | *Sub-totals* | *Totals* |
|---|---|---|---|---|---|---|
| | | + | — | 0 | | |
| Thalamus (12 cats) | + | 34 | 4 | 41 | 79 | |
| | — | 4 | 3 | 2 | 9 | |
| | 0 | 4 | 0 | 6 | 10 | 98 |
| Thalamus (3 rats) | + | 3+1* | 0 | 0 | 4 | |
| | — | 0 | 0 | 0 | 0 | |
| | 0 | 1 | 0 | 3 | 4 | 8 |
| Pericruciate cortex (3 cats) | + | 8 | 0 | 3 | 11 | |
| | — | 0 | 0 | 0 | 0 | |
| | 0 | 1 | 0 | 1 | 2 | 13 |
| Pericruciate cortex (2 rats) | + | 6 | 0 | 0 | 6 | |
| | — | 0 | 0 | 0 | 0 | |
| | 0 | 0 | 0 | 0 | 0 | 6 |
| Hypothalamus (13 cells in 3 cats and 7 cells in 2 rats) | + | 13 | 0 | 0 | 13 | |
| | — | 0 | 0 | 0 | 0 | |
| | 0 | 1 | 0 | 6 | 7 | 20 |
| Brain-stem (3 cats) | + | 7 | 0 | 3 | 10 | |
| | — | 0 | 0 | 0 | 0 | |
| | 0 | 2 | 0 | 0 | 2 | 12 |
| Totals | | 85 | 7 | 65 | 157 | 157 |

*ACh was not tested on this cell.
+, excitation; —, inhibition; 0, no effect.

**Table 2** Effect of iontophoretic or intravenous atropine on excitation of cortical and thalamic neurones in 7 cats and 2 rats

| | Effect of atropine (Atr)* | | | | | | |
|---|---|---|---|---|---|---|---|
| *Agonist* | *a* | *b* | *c* | *d* | *e* | *f* | |
| ACh | NT | — | — | — | — | — | |
| Li+ | — | — | 0 | — | — | NT | |
| Glut or DLH | NT | NT | NT | — | 0 | 0 | |
| *Atagonist* | | | *Number of cells* | | | | *Totals* |
| Atr 10–50 × $10^{-9}$ A | 4 | 5 | 4 | 2 | 1 | 3 | 19 |
| Atr 0.5 mg/kg i.v. | 0 | 2 | 0 | 0 | 0 | 0 | 2 |

*Antagonism of excitation by atropine (Atr) is indicated (—); no effect is indicated (0); NT indicates that a particular agonist was not tested on these cells.
Glut, L-glutamic acid; DLH, DL-homocysteic acid

only 8 neurones did one agent excite while the other inhibited and all of these were in the thalamus.

The time course of excitation by $Li^+$ was often, but not invariably, faster than that observed on Renshaw cells but effects of $Li^+$ and, to a lesser extent, of ACh were variable when tested repeatedly on the same cell.

### *The effect of atropine on excitation of supraspinal neurones*

Iontophoretic and, in two cats, intravenous administration of atropine on responses to ACh, $Li^+$ and excitant amino acids was examined in neurones in the cerebral cortex and thalamus of cats and rats (Table 2). The variability in the responses to $Li^+$ precluded many successful tests. In order to ascertain whether the effects of atropine were specific for ACh receptors we would have wished to have tested the effects of atropine on responses to ACh, $Li^+$ and an excitant amino acid on the same cell, but this was rarely possible, despite intensive efforts. Table 2 shows that iontophoretic atropine reduced the effect of ACh in the 15 cells tested and reduced the excitant effect of $Li^+$ on 12 of the 16 neurones examined. On 4 neurones, the action of atropine was selective for ACh receptors, leaving responses to excitant amino acids unchanged (Table 2, columns e, f), but on 2 cells (Table 2, column d) atropine did not have a selective action.

## Discussion

These experiments have failed to substantiate our original working hypothesis that lithium ions might impair cholinergic transmission by blocking the uptake of choline into cholinergic terminals in the central nervous system. Instead, our study indicated that cholinergic transmission is facilitated, rather than impaired.

The facilitation of cholinergic transmission was demonstrated on the Renshaw cell where $Li^+$ not only facilitated transmission at the cholinergic synapses of motor axon collaterals but also elevated the background discharge rate known to be blocked by the acetylcholine antagonist atropine (Curtis & Ryall, 1966b). The fact that the discharge evoked by the iontophoretic administration of ACh was usually reduced indicates that the facilitation of transmission was due to a presynaptic action causing an increased release of excitatory transmitter. Similar depressant effects of $Li^+$ on ACh responses have been demonstrated on mammalian sympathetic ganglion cells (Pappano & Volle, 1967) and on ganglion cells in *Aplysia* (Ono, Sato & Muruhashi, 1974). The depressant effects could be due to a postsynaptic membrane stabilizing action brought about by an increased potassium conductance (Partidge & Thomas, 1974). However, in the rat hippocampus (Segal, 1974) iontophoretic $Li^+$ did not antagonize the excitatory effect of ACh but reduced the inhibitory action of noradrenaline and 5-hydroxytryptamine but not that of $\gamma$-aminobutyric acid.

Dihydro-$\beta$-erythroidine is a potent and selective ACh antagonist at postsynaptic nicotinic receptors on Renshaw cells (Curtis & Ryall, 1966a,b). It reduced not only the synaptically evoked discharges and responses to ACh but also reduced the discharges caused by $Li^+$. It therefore seems reasonable to propose that a part, at least, of the facilitatory action of $Li^+$ on Renshaw cells was attributable to a presynaptic mechanism causing the release of the endogenous transmitter ACh. The time course of the facilitation by $Li^+$ was often although not invariably slow, which would be consonant with a slow intracellular accumulation of $Li^+$ within the terminals. This slow build-up might account for the variability in the effects observed on different cells and might be critically dependent upon the precise location of the ejecting micropipette. A presynaptic effect, causing the release of ACh, is in agreement with observations made at the neuromuscular junction (Kelly, 1968; Carmody & Gage, 1973; Crawford, 1975) and on resting release of ACh from rat cortical slices (Vizi, Illés, Rónai & Knoll, 1972).

However, it is difficult to conceive a mechanism by which $Li^+$ would have an effect on presynaptic and not on postsynaptic structures. The fact that responses to ACh were reduced by $Li^+$ supports this postulate. However, it must be concluded that the facilitation exerted on presynaptic structures must more than counterbalance the depressant effect observed postsynaptically, possibly due to a greater dependence of the release mechanism on small ionic changes brought about by the accumulation of $Li^+$.

A question which arose from these observations was whether $Li^+$ might have a similar effect on all presynaptic terminals, including non-cholinergic as well as cholinergic terminals. Since $Li^+$ did not excite spinal interneurones, which were not cholinoceptive and therefore presumably were not innervated by cholinergic terminals, the evidence is suggestive that $Li^+$ has a predilection for cholinergic rather than non-cholinergic terminals.

The mixed inhibitory-excitatory action of hemicholinium on Renshaw cells has been reported previously (Quastel & Curtis, 1965), but the fact that it also excites non-cholinoceptive interneurones indicates that the excitatory action has no relation to cholinergic transmission.

The predilection of $Li^+$ for cholinergic synapses in the central nervous system is also supported by the observations made on supraspinal neurones in the cerebral cortex, thalamus, hypothalamus and brainstem. At these sites an excitatory action of $Li^+$ was usually associated with an excitatory action of ACh, although the reverse situation was less evident, possibly due to a lower efficacy of $Li^+$ compared with ACh. On a few cells in the thalamus, probably located in the nucleus reticularis (Ben-Ari, Dingledine, Kanazawa & Kelly, 1976) a depressant effect of both $Li^+$ and ACh was observed, again supporting the postulated relationship between the action of $Li^+$ and cholinergic synapses.

Although relatively few in number, there were some instances of a lack of correlation between the action of $Li^+$ and ACh at supraspinal levels and we must not be trapped into the probably false conclusion that $Li^+$ only affects cholinergic synapses in the central nervous system. It seems unlikely that any mechanism could be postulated by which such an absolute specificity could occur and there are numerous reports in the literature of changes in other neurotransmitter systems after $Li^+$ administration. However, our results do indicate a degree of specificity at cholinergic locations and it is possible that alterations in the function of other neurotransmitter systems may arise secondarily from changes in cholinergic transmission. It is unlikely that the effects observed in the present series of experiments were due to secondary effects on other transmitters because the iontophoretic technique ensures a degree of localization of the effects not attainable by systemic administration.

The antagonism of the effects of $Li^+$ on supraspinal neurones by atropine supports the conclusion that they were often mediated via a release of ACh.

$Ba^{2+}$ selectively excites cholinoceptive neurones in the cerebral cortex (Krnjević, Pumain & Renaud, 1971). However, this effect is claimed to be postsynaptic because it was not blocked by atropine and therefore appears to be different from that of $Li^+$ in the present experiments. However, $Ba^{2+}$ stimulates the secretion of catecholamines from the adrenal medulla by a mechanism which differs from that of ACh in being independent of external $Ca^{2+}$ (Douglas & Rubin, 1964), and release of ACh by $Li^+$ at the neuromuscular junction can also occur in the absence of external $Ca^{2+}$ (Carmody & Gage, 1973). It is therefore possible that the presumed effects of $Li^+$ on ACh release in the present investigation may be similar to that of $Li^+$ at the neuromuscular junction and to that of $Ba^{2+}$ on catecholamine release in the adrenal medulla.

In conclusion, it is believed that this study indicates that the central actions of lithium may be chiefly due to a facilitating effect on transmitter release, although there was also some evidence of a postsynaptic depressant action on cell excitability of less importance. It is also concluded that the presynaptic effect may be more pronounced on cholinergic than non-cholinergic terminals. It is suggested, tentatively, that this mechanism could be the basis of the therapeutic efficacy of $Li^+$ in the treatment of manic-depression. In this condition, the slow onset of the effect of $Li^+$ could be attributable to a slow accumulation of $Li^+$ within the terminals, limited by the low plasma levels that can be tolerated. However, the occurrence of a relatively non-reversible mechanism (Lingsch & Martin, 1976) which does not depend on relatively simple ionic disturbances cannot be discounted in the explanation both of the therapeutic effect of $Li^+$ and the postulated facilitation of transmitter release.

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# CATECHOL-SUBSTITUTED PHENOXYPROPANOLAMINES: ADRENOCEPTOR ACTIVITY IN THE ANAESTHETIZED CAT

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1 The pharmacological actions of racemic noradrenaline, adrenaline, isoprenaline and *N-t*-butylnoradrenaline have been compared with those of their corresponding derivatives containing an oxymethylene (OXY) link between the ring and ethanolamine side chain.

2 The compounds were tested in the anaesthetized cat for their ability to produce positive chronotropic effects, bronchodilator actions, changes in perfusion pressure in the perfused hind limb and decreases in soleus muscle contractions.

3 All the OXY-derivatives were potent $\beta$-adrenoceptor agonists. The inclusion of the oxymethylene link promotes selectivity for $\beta_1$- as opposed to $\beta_2$-adrenoceptor activity.

4 In comparison with the parent compounds, the OXY-derivatives of adrenaline and noradrenaline had very weak $\alpha$-adrenoceptor stimulant effects.

## Introduction

From a structural standpoint $\beta$-adrenoceptor antagonists fall into two main groups, ring substituted phenylethanolamines (e.g. dichloroisoprenaline, sotalol etc.) and ring substituted phenoxypropanolamines which contain an oxymethylene link between the ring and the ethanolamine side chain (e.g. propranolol, oxprenolol, pindolol etc.).

Although adrenoceptor agonists based on the phenylethanolamine nucleus have been used in numerous studies, little work has been done with phenoxypropanolamine derivatives. In 1970 Ablad, Brögard & Corrodi showed that the phenoxypropanolamine derivatives of isoprenaline and orciprenaline possessed affinity for adrenoceptor sites, since both agents produced $\beta$-adrenoceptor mediated positive chronotropic effects in anaesthetized guinea-pigs.

In the present study this work has been extended. Phenoxypropanolamine derivatives of noradrenaline, adrenaline, isoprenaline and *N-t*-butylnoradrenaline (Figure 1) have been synthesized and tested for their pharmacological actions in the anaesthetized cat. Details of the chemistry of these compounds will be reported elsewhere. For convenience these compounds will be termed OXY-derivatives of their respective parent compounds. In all experiments racemic OXY-derivatives and parent compounds have been compared in order to determine how the inclusion of the oxymethylene link affects the potency and selectivity of the agents for adrenoceptor mediated effects.

## Methods

Cats of either sex weighing 1.2–5 kg were anaesthetized by the intraperitoneal injection of $\alpha$-chloralose (80 mg/kg) and sodium pentobarbitone (6 mg/kg). In all experiments arterial blood pressure (1 mmHg $\approx$ 133 Pa) was monitored from a cannulated carotid artery with a Statham (P23DC) pressure transducer, and heart rate measured with a Grass tachograph (7P4) triggered by the arterial pulse.

In experiments where the cardiac chronotropic actions of the compounds were assessed, the animals were bilaterally vagotomized and artificially respired at a rate of 20 breaths/min and a stroke volume of 15 ml/kg body weight.

These respiratory parameters were also used when testing the bronchodilator actions of the compounds. In these studies intratracheal pressure was monitored with a pressure transducer connected to a side arm of the tracheal cannula as described by McCulloch, Proctor & Rand (1967). Bronchoconstriction was induced by the intravenous infusion of 5-hydroxytryptamine (8.9–58.2 $\mu g\,kg^{-1}\,min^{-1}$ in different ex-

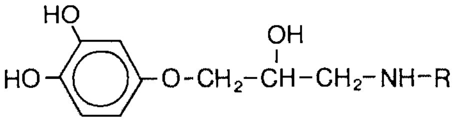

**Figure 1** General formula of the racemic OXY-compounds used in the present study. Derivatives of noradrenaline (R=H), adrenaline (R=$CH_3$), isoprenaline (R=$CH(CH_3)_2$) and *N-t*-butyl-noradrenaline (R=$C(CH_3)_3$) were used.

periments). When the bronchoconstriction had stabilized, the amines were injected and their bronchodilator actions monitored as reductions in intratracheal pressure. As soon as maximal bronchodilator effects had been attained the 5-hydroxytryptamine infusion was stopped and the lungs hyperinflated for three breaths in order to prevent atelectasis. If required, gallamine (3.4 µmol/kg) was injected intravenously to prevent spontaneous respiratory movements.

In experiments where hind-limb resistance was measured, blood was taken from the left femoral artery and pumped into the right femoral artery with a Watson-Marlow (Type 200) constant flow inducer. Perfusion pressure in the limb was measured with a pressure transducer (Statham P23DC) in the post-pump section of the circuit. The flow rate was initially adjusted so that perfusion pressure was approximately the same as the systemic arterial pressure. This flow rate was then maintained throughout the rest of the experiment. Drugs were injected in volumes <50 µl into the pre-pump circuit. In these experiments the cats were given bethanidine (22 µmol/kg; 6 mg/kg i.v.) to reduce sympathetic reflexes and heparin (1200 u/kg) as an anticoagulant. The perfused hind-limb was also denervated by sectioning the femoral and sciatic nerves. As a routine the animals were given 5 ml of a 10% dextran 40 solution intravenously to make up for the dead space in the external circuit.

The soleus muscle was prepared for the recording of contractions as described by Bowman & Nott (1970). The muscle was stimulated at a frequency of 8 Hz for 1 s once every 10 seconds. Sympathomimetic-induced decreases in the tension and fusion of the sub-tetanic contractions were monitored as described by Nott & Raper (1972) using a Grass FT10C force-displacement transducer.

Unless otherwise stated in the text, actions of all the amines were monitored by the use of cumulative dose-response curves. Drugs were injected intravenously into a cannulated brachial vein. In each experiment constant curves to isoprenaline were first obtained and thereafter responses to pairs of corresponding parent and OXY-compounds assessed in a randomized fashion. Control curves to isoprenaline were reassessed between each pair of drugs tested.

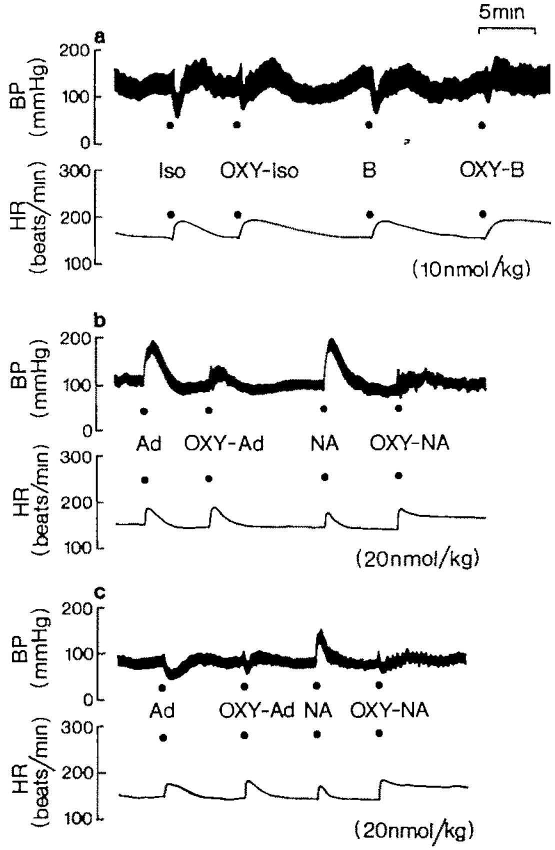

**Figure 2** Recordings of arterial blood pressure (BP) and mean heart rate (HR) in anaesthetized cats. In (a) responses to isoprenaline (Iso), OXY-isoprenaline (OXY-Iso), *N-t*-butylnoradrenaline (B) and OXY-*N-t*-butylnoradrenaline (OXY-B) and in (b) and (c) responses to adrenaline (Ad), OXY-adrenaline (OXY-Ad), noradrenaline (NA) and OXY-noradrenaline (OXY-NA). Trace (b) shows responses before, and trace (c) responses after α-adrenoceptor blockade with phentolamine.

Drugs used were (±)-isoprenaline hydrochloride, (±)-noradrenaline hydrochloride, (±)-adrenaline (Sigma), (±)-*N-t*-butylnoradrenaline mesylate (Stirling Winthrop), the racemic oxymethylene derivatives of the above catecholamines (Department of Chemistry, Victorian College of Pharmacy), phentolamine mesylate (Ciba), bunitrolol hydrochloride (Kö1366, Boehringer Ingelheim), 5-hydroxytryptamine (serotonin) creatinine sulphate (Sigma), gallamine triethiodide (May & Baker) and bethanidine sulphate (Burroughs Wellcome). Solutions of the catecholamines were freshly prepared in 0.01 mol/l HCl and suitable dilutions made up in 0.9% w/vNaCl solution (saline) containing 20 µg/ml ascorbic acid.

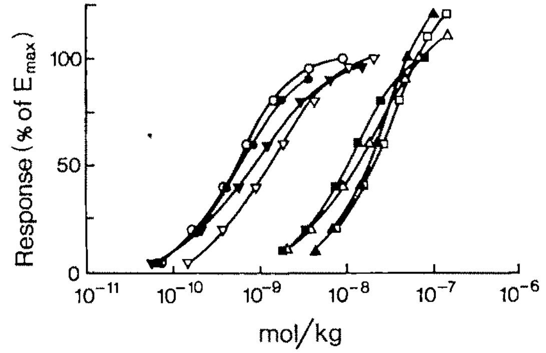

**Figure 3** Mean dose-response curves showing the positive chronotropic effects of noradrenaline (□), OXY-noradrenaline (■), adrenaline (▲), OXY-adrenaline (△), isoprenaline (○), OXY-isoprenaline (●), *N-t*-butylnoradrenaline (▼), and OXY-*N-t*-butylnoradrenaline (▽) in anaesthetized cats Responses are expressed in terms of the maximal response produced by isoprenaline (=100% or $E_{max}$). For isoprenaline $n=10$, and for the remaining compounds $n=5$

In all experiments traces were recorded on an ink-writing Grass Model 7C polygraph.

## Results

### *General cardiovascular activity*

In 5 pilot experiments responses to single injections of the compounds were assessed in bilaterally vagotomized and anaesthetized cats in which arterial blood pressure and heart rate were recorded. Isoprenaline, *N-t*-butylnoradrenaline and their respective OXY-derivatives produced vasodepressor responses and increases in heart rate which were completely abolished by the prior injection of the β-adrenoceptor antagonist, bunitrolol (3.5 μmol/kg; 1 mg/kg). At equivalent mol/kg doses all four agonists produced similar positive chronotropic effects, whereas the vasodepressor actions of the OXY-derivatives were smaller than those of the parent compounds (Figure 2a).

Adrenaline and noradrenaline and their respective OXY-derivatives produced increases in heart rate and vasopressor actions. Although similar cardiac responses were obtained with the same mol/kg doses of all four compounds, the vasopressor responses to OXY-adrenaline and OXY-noradrenaline were much smaller than those produced with the parent compounds (Figure 2b). After β-adrenoceptor blockade with bunitrolol (3.5 μmol/kg), the positive chronotropic actions of the compounds were abolished; pressor responses to adrenaline were slightly increased, those to noradrenaline little changed, and those to the OXY-derivatives reduced. Phentolamine (5.3 μmol/kg; 2 mg/kg), caused a slight reduction in resting blood pressure, antagonized the pressor response to noradrenaline and produced vasomotor reversal with adrenaline, OXY-adrenaline and OXY-noradrenaline (Figure 2c). These vasodepressor responses, which were unmasked after α-adrenoceptor blockade, were abolished after bunitrolol (3.5 μmol/kg) administration.

The above results suggested that the cardiovascular actions of the OXY-compounds involved effects at both α- and β-adrenoceptors, and on this basis more detailed studies were performed to assess the actions of the drugs in cardiac, vascular, bronchial and skeletal muscle.

### *Cardiac activity*

Figure 3 shows the mean cumulative dose-response curves for the positive chronotropic effect of the parent compounds and their OXY-derivatives in vagotomized animals. Dose-response lines had a similar slope with all the compounds tested. Maximal responses to *N-t*-butylnoradrenaline and all the OXY-derivatives tested were similar to that of isoprenaline, while slightly greater values were obtained with adrenaline and noradrenaline. After maximal chronotropic effects had been obtained the times to half-return of heart rate to control levels varied within the group of compounds tested; for the parent compounds and OXY-adrenaline these times fell within the range of 3–10 min, for OXY-isoprenaline 3–17 min and for OXY-noradrenaline and OXY-*N-t*-butylnoradrenaline >30 minutes. The longer lasting responses obtained with the latter compounds can be seen in Figure 2.

In each experiment the doses of the compounds required to produce 50% of the maximal effect of isoprenaline ($ED_{50}$ values) were interpolated. These values were used to calculate mean $ED_{50}$ values and relative potencies with respect to isoprenaline (Table 1). When comparing the $ED_{50}$ values of each parent compound and its OXY-derivative it was found that OXY-noradrenaline was approximately twice as potent as noradrenaline in producing positive chronotropic actions. With each of the remaining pairs of compounds there was no significant difference between the $ED_{50}$ value of the parent drug and its corresponding OXY-derivative ($P > 0.05$).

### *Vascular activity*

In these experiments vasoconstrictor and vasodilator responses were assessed from changes in perfusion pressure induced by single bolus injections of the compounds. Within each experiment dose-response curves were constructed, each point being the mean

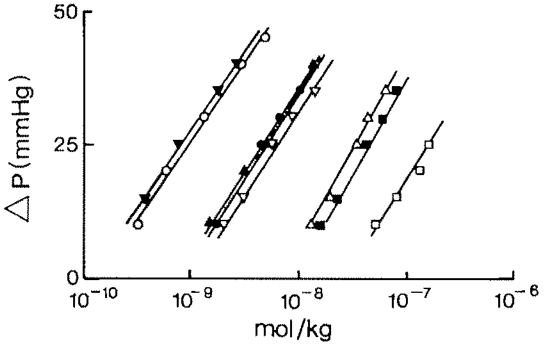

**Figure 4** Mean dose-response lines showing decrease in perfusion pressure in the perfused hind-limb of anaesthetized cats in the presence of α-adrenoceptor blockade with phentolamine Symbols for the various drugs used are indicated in Figure 3. Lines show mean results from 13 experiments with isoprenaline and 4 to 9 experiments with each of the remaining drugs except noradrenaline. The responses to the latter agent were obtained in the one experiment in which uncomplicated vasodilator responses were obtained.

obtained from 3 or 4 randomized responses to a given dose of a compound.

Vasoconstrictor actions due to α-adrenoceptor mediated effects of the compounds were monitored in preparations following β-adrenoceptor blockade with bunitrolol (3.5 µmol/kg). Under these conditions increases in perfusion pressure were only obtained with adrenaline, noradrenaline, OXY-adrenaline and OXY-noradrenaline. In comparison with the parent compounds, which produced dose-related vasoconstrictor actions, the OXY-derivatives produced only weak activity. Thus, at 20 nmol/kg adrenaline and noradrenaline produced mean increases in perfusion pressure (±s.e. mean, $n=5$) of 80 ± 10 and 76 ± 12 mmHg respectively. At this dose level the mean responses ($n=5$) to OXY-adrenaline and OXY-noradrenaline were 6.0 ± 1.7 and 4.5 ± 1.5 mmHg. In comparison with the parent compounds the dose-response curves to the OXY-derivatives were very shallow, and within individual experiments there was a poor dose-response relationship.

The ability of the drugs to produce decreases in perfusion pressure were assessed in the presence of α-adrenoceptor blockade with phentolamine. An initial dose of 5.3 µmol/kg was given and thereafter doses of 2.7 µmol/kg were administered at hourly intervals throughout the experiment. In these experiments no attempt was made to determine the maximal vasodilator actions of the compounds, since in pilot studies it was found that the reproducibility of responses was adversely affected after doses of the drugs that produced near maximal or supramaximal effects.

With the exception of noradrenaline all the compounds produced dose-related decreases in perfusion pressure (Figure 4). With the former compound weak vasodilator actions were only seen in

**Table 1** Doses of the compounds and their relative potencies with respect to isoprenaline

| | Heart | | Vessels | | Bronchi | | Soleus | |
|---|---|---|---|---|---|---|---|---|
| | *ED*$_{50}$ | *RP* | *ED*$_{25}$ | *RP* | *ED*$_{50}$ | *RP* | *ED*$_{50}$ | *RP* |
| Noradrenaline | 20.7 | 37.7 | 167 | 225 | 279 | 161 | 147 | 399 |
| | (1.6) | (4.8) | – | – | (74) | (40.2) | (16.6) | (48.2) |
| OXY-noradrenaline | 10.3 | 19.2 | 51.6 | 89.8 | 76.2 | 45.5 | 17.7 | 49.0 |
| | (2.2) | (5.2) | (9.0) | (6.1) | (13.7) | (9.6) | (1.4) | (3.4) |
| Adrenaline | 18.7 | 33.6 | 5.00 | 6.7 | 12.4 | 6.3 | 1.74 | 4.8 |
| | (3.9) | (9.0) | (2.3) | (1.4) | (1.8) | (1.2) | (0.17) | (0.5) |
| OXY-adrenaline | 13.2 | 23.1 | 43.1 | 64.0 | 108 | 49.2 | 23.1 | 62.8 |
| | (2.1) | (3.2) | (13.6) | (9.6) | (20.4) | (7.4) | (1.5) | (8.6) |
| Isoprenaline | 0.51 | 1.0 | 0.83 | 1.0 | 1.89 | 1.0 | 0.31 | 1.0 |
| | (0.10) | – | (0.23) | – | (0.16) | – | (0.04) | – |
| OXY-isoprenaline | 0.58 | 1.1 | 4.52 | 5.4 | 8.15 | 5.2 | 0.85 | 4.1 |
| | (0.18) | (0.17) | (1.13) | (1.2) | (2.21) | (1.5) | (0.11) | (1.4) |
| *N*-*t*-butyl-noradrenaline | 0.84 | 1.8 | 0.80 | 0.82 | 1.39 | 0.83 | 0.16 | 0.73 |
| | (0.39) | (0.33) | (0.37) | (0.11) | (0.27) | (0.09) | (0.01) | (0.18) |
| OXY-*N*-*t*-butyl-noradrenaline | 1.33 | 3.6 | 6.2 | 7.1 | 3.88 | 3.4 | 0.61 | 3.0 |
| | (0.43) | (2.1) | (1.6) | (1.6) | (0.72) | (0.5) | (0.07) | (1.0) |

Mean doses of the compounds (µmol/kg) required to produce 50% of the maximal effect (ED$_{50}$) in the heart, bronchi and soleus muscle and a 25 mmHg reduction in the perfusion pressure in the hind-limb (ED$_{25}$). Mean relative potencies (RP) with respect to isoprenaline are also shown. Standard errors of the mean are shown in parentheses. Values obtained from 4–9 experiments with each drug except noradrenaline in the perfused hind-limb, where values were obtained in the one experiment in which uncomplicated vasodilator effects were monitored.

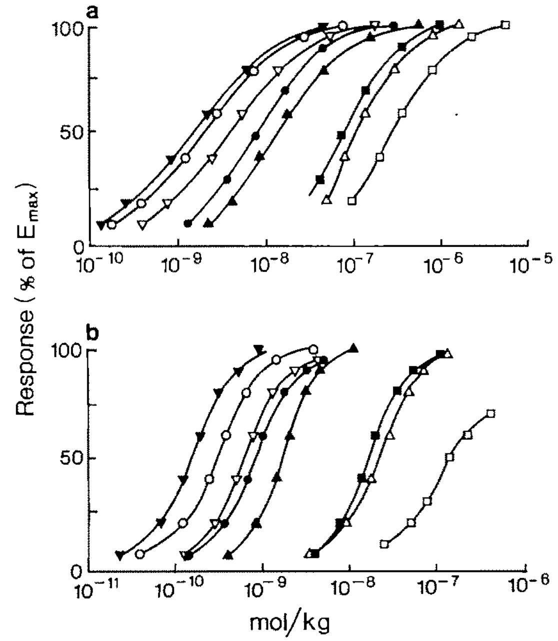

**Figure 5** Mean dose-response curves for (a) bronchodilator effects and (b) reduction in the tension of sub-tetanic contractions of the soleus muscle in anaesthetized cats. Symbols for the various drugs used are shown in Figure 3. Responses are expressed in terms of the maximal effects produced with isoprenaline (=100%). For isoprenaline $n=12$ in (a) and 8 in (b). With the remaining drugs $n=4$–8.

one of five experiments. The results from this single experiment with noradrenaline are shown in Figure 4. In the remaining studies vasoconstrictor actions dominated the vascular effects produced. Increasing the dose of phentolamine reduced these vasoconstrictor effects but failed to unmask vasodilator activity. The dose of phentolamine that could be used was limited by its own vascular effects; these reduced the resting perfusion pressure to such an extent that little or no vasodilatation was apparent even when drugs such as isoprenaline were used.

Since maximal vasodilator effects were not monitored, relative potencies in each experiment were calculated from doses producing a 25 mmHg reduction of perfusion pressure (Table 1). OXY-adrenaline, OXY-isoprenaline and OXY-*N*-*t*-butylnoradrenaline were all significantly less potent ($P<0.05$) than their respective parent compounds in producing decreases in perfusion pressure. OXY-noradrenaline was more potent than noradrenaline as a vasodilator ($P<0.05$). As in the experiments where cardiac chronotropic actions were measured, OXY-noradrenaline and OXY-*N*-*t*-butylnoradrenaline produced longer lasting vasodilator actions than those found with the other compounds tested.

### *Bronchodilator activity*

As found by previous authors (Rodger, 1974; Malta & Raper, 1976) the bronchoconstriction produced by a given infusion rate of 5-hydroxytryptamine was remarkably constant within each experiment. In the present studies reproducible bronchoconstrictor responses to 5-hydroxytryptamine and bronchodilator responses to all the compounds except OXY-noradrenaline and OXY-*N*-*t*-butylnoradrenaline could be obtained at 45 min intervals. Responses to 5-hydroxytryptamine were reduced 45 min after dose-response curves to the latter compounds had been established. This probably reflects their long duration of action, since after a further 45 min responses to 5-hydroxytryptamine returned to control levels.

All the compounds tested produced dose-related bronchodilator effects; similar maximal responses were produced and dose-response lines were closed to parallel for all compounds (Figure 5a).

In one of the initial experiments in which noradrenaline was tested, the amine produced a dose-related increase in intratracheal pressure, and in another the lower cumulative doses produced a bronchodilator action, while higher doses reversed this effect and induced a weak bronchoconstrictor effect. In both cases the bronchoconstrictor effects were abolished by the prior administration of phentolamine (5.3 μmol/kg). In the remaining 8 experiments noradrenaline produced only bronchodilator activity, maximal responses and the slope of the dose-response lines being similar to those produced by isoprenaline. In 4 of these experiments responses to noradrenaline alone were first monitored and thereafter responses were assessed in the presence of phentolamine (5.3 μmol/kg). There was no significant difference in the $ED_{50}$ doses or the relative potencies of noradrenaline with respect to isoprenaline in the presence or absence of $\alpha$-adrenoceptor blockade ($P>0.05$). A similar finding was obtained in one experiment in which the effects of adrenaline were monitored in the absence and presence of phentolamine. In these experiments phentolamine itself slightly reduced the bronchoconstrictor actions of 5-hydroxytryptamine. The infusion rate of the latter agent was therefore increased so that the bronchodilator effects of the catecholamines could be assessed against a constant degree of background constriction.

The mean $ED_{50}$ doses of the compounds and their relative potencies with respect to isoprenaline are shown in Table 1. Since $\alpha$-adrenoceptor blockade did not affect these values for noradrenaline, results obtained in the presence and absence of phentolamine have been used for calculating the means. In terms of bronchodilator activity OXY-noradrenaline was significantly more potent than noradrenaline ($P < 0.05$) and OXY-adrenaline, OXY-isoprenaline and OXY-*N*-*t*-butylnoradrenaline were significantly less potent than their respective parent compounds ($P < 0.05$).

*Soleus muscle contractions*

The ability of $\beta$-adrenoceptor agonists to decrease the tension and fusion of sub-tetanic contractions of the cat soleus muscle has been used to assess potential tremorogenic actions of sympathomimetic bronchodilators (Bowman & Nott, 1970; Apperley, Daly & Levy, 1976). All the compounds used in the present study produced the characteristic decrease in soleus muscle contractions. With the exception of noradrenaline all compounds produced a similar maximal response to that obtained with isoprenaline and dose-response lines were close to parallel (Figure 5b). Mean $ED_{50}$ doses are shown in Table 1. At the high doses of noradrenaline required to produce effects on soleus muscle contractions, the intense $\alpha$-adrenoceptor mediated vasoconstrictor effects probably interfere with the results obtained, since in the presence of phentolamine or phenoxybenzamine there is a small shift to the left of dose-response curves to the amine and maximal responses are slightly increased (Raper, unpublished observations). However, complete abolition of pressor responses to the amine were impossible to achieve with doses of the $\alpha$-adrenoceptor antagonists which did not adversely affect muscle contractility through probably non-specific actions. As in the bronchi and hind-limb the OXY-derivatives of adrenaline, isoprenaline and *N*-*t*-butyl noradrenaline were significantly less potent than their respective parent compounds ($P < 0.05$) while OXY-noradrenaline was more potent than noradrenaline ($P < 0.05$). As in the other tissues responses to OXY-noradrenaline and OXY-*N*-*t*-butylnoradrenaline were longer lasting than those of the other compounds.

**Discussion**

In the present study a series of catecholamines with and without an oxymethylene link between the ring and ethanolamine side chain have been compared for their $\alpha$- and $\beta$-adrenoceptor activity in the anaesthetized cat. The results indicate that the inclusion of the oxymethylene link leads to a retention of agonistic actions at $\beta$-adrenoceptor sites and a substantial reduction in $\alpha$-adrenoceptor mediated activity. In terms of potency, both the parent compounds and their OXY-derivatives have a similar activity for $\beta_1$-receptor mediated actions in the heart. With the exception of OXY-noradrenaline, the remaining derivatives are less potent than their parent compounds in producing $\beta_2$-receptor mediated effects in bronchial, vascular and skeletal muscle.

The selectivity of action of agonists at $\beta_1$- and $\beta_2$-receptor sites is best indicated by calculating selectivity ratios with respect to an internal standard such as isoprenaline (Bowman & Raper, 1976). If cardiac stimulant actions are used to represent $\beta_1$- and bronchodilator effects $\beta_2$-receptor mediated responses, the selectivity ratios [i.e. dose-ratio (heart) : dose-ratio (bronchi)] found for noradrenaline, adrenaline, isoprenaline and *N*-*t*-butylnoradrenaline are 0.23, 5.33, 1.0 and 2.17 respectively. The selectivity ratios for the corresponding OXY-derivatives are 0.42, 0.47, 0.21 and 1.05 respectively. Thus within the parent compounds noradrenaline is $\beta_1$-selective, and adrenaline and *N*-*t*-butylnoradrenaline are $\beta_2$-receptor

**Table 2** Selectivities of compounds for $\beta_1$- and $\beta_2$-adrenoceptor mediated actions

| | *Hearts . Vessels* | | *Heart : Bronchi* | | *Heart : Soleus* | |
|---|---|---|---|---|---|---|
| | *RP* | *$ED_{25}$* | *RP* | *$ED_{50}$* | *RP* | *$ED_{50}$* |
| Noradrenaline | 0.16 | 0.12 | 0.23 | 0.07 | 0.09 | 0.14 |
| OXY-noradrenaline | 0.21 | 0.19 | 0.42 | 0.13 | 0.39 | 0.58 |
| Adrenaline | 5.01 | 3.7 | 5.33 | 1.51 | 7.0 | 10.75 |
| OXY-adrenaline | 0.36 | 0.30 | 0.47 | 0.12 | 0.36 | 0.57 |
| Isoprenaline | 1.0 | 0.61 | 1.0 | 0.27 | 1.0 | 1.64 |
| OXY-isoprenaline | 0.20 | 0.13 | 0.21 | 0.07 | 0.27 | 0.68 |
| *N*-*t*-butyl noradrenaline | 2.19 | 1.05 | 2.17 | 0.60 | 2.46 | 5.25 |
| OXY-*N*-*t*-butyl-noradrenaline | 0.50 | 0.21 | 1.05 | 0.34 | 1.20 | 2.18 |

$\beta_1 : \beta_2$ Receptor selectivities based on (a) the mean relative potencies (RP) of the compounds with respect to isoprenaline in the various tissues, and (b) mean $ED_{50}$ doses in heart, bronchi and soleus, and doses to produce a 25 mmHg decrease in perfusion pressure in the hind-limb. Values greater than unity indicate $\beta_2$-receptor selectivity and values less than unity $\beta_1$-selective actions.

selective in their effects. The inclusion of the oxymethylene link appears to promote $\beta_1$-receptor selective actions, since the selectivity ratios for OXY-noradrenaline, OXY-adrenaline and OXY-isoprenaline are less than unity. This effect is also apparent with OXY-*N*-*t*-butylnoradrenaline which displays non-selective actions as opposed to the $\beta_2$-selectivity of the parent compound. The trend towards $\beta_1$-selective actions is also apparent in the OXY-derivatives if selectivity ratios are calculated using dose-ratios for $\beta_2$-receptor mediated actions in the hind-limb or soleus muscle, or if selectivity is assessed using the actual doses of the compounds ($ED_{50}$ values) required to produce effects in the various tissues (Table 2).

In addition to changes in selectivity, the inclusion of the OXY-methylene link also affects the duration of action of the compounds. This is especially noticeable with OXY-noradrenaline and OXY-*N*-*t*-butylnoradrenaline which have a much longer duration of action than their respective parent compounds. Whether or not the longer lasting actions are due to a lower affinity of the OXY-derivatives for uptake processes or metabolizing enzymes requires further elucidation.

Although a great deal of information is available regarding the structural requirements for agents displaying $\beta_2$-adrenoceptor selective actions (Brittain, Jack & Ritchie, 1970) little comparable work has been done for $\beta_1$-selective activity. The finding that catecholamines of the phenoxypropanolamine type display selectivity for $\beta_1$- as opposed to $\beta_2$-adrenoceptor mediated effects and are virtually devoid of $\alpha$-adrenoceptor stimulant actions is of interest in this regard.

Drugs which produce cardiac stimulation in the absence of marked vascular changes may be of potential use in treating low cardiac output states. Further work with agonists of the phenoxypropanolamine type may therefore be of interest.

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# CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE AND LEUCOCYTE CHEMOTAXIS *in vivo*

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1 The effect of local elevation of leucocyte cyclic adenosine 3′,5′-monophosphate (cyclic AMP) content on the continued migration of leucocytes to a site of acute inflammation was studied in the pleural cavity of rats.

2 Leucocyte cyclic AMP levels were elevated by injecting with the irritant into the pleural cavity dibutyryl cyclic AMP alone or with theophylline.

3 The treatments both produced a marked reduction in leucocyte migration into a pleural reaction induced by immediate hypersensitivity, but had no effect on cell numbers in pleurisy induced either by pyrophosphate or urate crystals.

## Introduction

The pleural cavity of rats has been shown to be a useful site for studying the effect of elevating endogenous leucocyte cyclic adenosine 3′,5′-monophosphate (cyclic AMP) levels on the progression of acute inflammation *in vivo* (Deporter, Capasso & Willoughby, 1976; Deporter, 1977; Deporter, Dieppe, Glatt & Willoughby, 1977). The inflammatory reactions studied included an immediate hypersensitivity (Arthus) reaction and inflammation induced by calcium pyrophosphate crystals, both of which have relevance to human arthritis (Willoughby, 1976). In the present study the same two models have been used to study the effect of local elevation of leucocyte cyclic AMP concentration on the continued migration of leucocytes to a site of acute inflammation.

## Methods

Male Wistar rats weighing 200–250 g were used. Pleurisy was induced by a mixture of pyrogen-free monoclinic and triclinic calcium pyrophosphate crystals as previously described (Deporter, Dieppe & Willoughby, 1976) or by immediate-hypersensitivity (reverse passive Arthus reaction) as described by Yamamoto, Dunn, Deporter, Capasso, Willoughby & Huskisson (1975). Briefly, bovine serum albumin was injected intravenously and 20–30 min later the animals were challenged intrapleurally with a purified rabbit antibody to bovine serum albumin. Pleurisy was also produced by injecting 1 ml of a solution of sodium biurate (10 mg/ml of saline) prepared according to the technique of Seegmiller, Howell & Malawista (1962).

To evaluate the effect of elevated leucocyte cyclic AMP content on the migration of more leucocytes into the pleural cavity in the three different pleural reactions, 2.45 mg dibutyryl cyclic AMP (Sigma) with and without 0.99 mg theophylline (BDH) was injected into the pleural cavity with the irritant. Both these drug treatments produce marked increases in the cyclic AMP content of leucocytes present in the pleural reactions under study (Deporter *et al.*, 1977; Deporter, 1977). Three hours after the onset of each reaction the animals were anaesthetized with ether and exanguinated via the carotid artery. Pleural exudates were withdrawn, blood-free, in siliconized Pasteur pipettes. After noting the volume of actual exudate in each animal, the pleural cavity was washed quickly with 2 ml of phosphate-buffered saline pH 7.4. Following brief agitation, the combined exudate and wash-out for each animal was sampled with a WBC pipette, diluted 1:20 with WBC diluent and counted within 30 minutes. Care was taken to count only samples free of WBC clumping within the WBC pipettes. WBC were counted 3 h after injection of the irritants, since by this time the three reactions studied were well advanced. Also, other experiments had shown that an effect on cyclic AMP levels in leucocytes by the drug treatments used could be maintained for 3 h but not longer (Deporter, unpublished results).

## Results

The results are shown in Table 1. Dibutyryl cyclic AMP alone or in combination with the cyclic AMP

**Table 1** Effect of local administration of dibutyryl cyclic AMP (2.45 mg) with or without theophylline (0.99 mg) on the migration of leucocytes into the pleural cavity of rats during three types of acute pleurisy

| *Type of pleurisy* | | *Total WBC* ($\times 10^6$) |
|---|---|---|
| Arthus-induced pleurisy | Control | 60.4 ± 3.7 |
| | Dibutyryl cyclic AMP | 39.2 ± 6.0* |
| | Dibutyryl cyclic AMP and theophylline | 36.4 ± 8.4* |
| Pyrophosphate pleurisy | Control | 33.8 ± 5.2 |
| | Dibutyryl cyclic AMP | 34.0 ± 5.1 |
| | Dibutyryl cyclic AMP and theophylline | 36.5 ± 7.1 |
| Urate pleurisy | Control | 28.2 ± 3.7 |
| | Dibutyryl cyclic AMP | 30.8 ± 3.1 |
| | Dibutyryl cyclic AMP and theophylline | 26.3 ± 4.9 |

* $P < 0.05$.

phosphodiesterase inhibitor theophylline significantly decreased leucocyte counts in intrapleural Arthus reactions. In contrast, in pleurisy produced by pyrophosphate or urate crystals, (Table 1) neither drug treatment produced a significant change in the numbers of migrating leucocytes. Moreover, neither drug treatment produced significant changes in exudate volume over control values in any of the three models studied (results not shown).

## Discussion

The mechanism by which a local elevation of leucocyte cyclic AMP content could reduce further leucocyte migration to a site of inflammation induced by immediate hypersensitivity is not known. However, since polymorphonuclear leucocytes (PMNs) engaged in phagocytosis of a variety of particles including antigen-antibody complexes release chemotatic factors for other PMNs (Tse & Phelps, 1970; Keller & Borel, 1971; Henson, 1972), it is conceivable that the elevated cyclic AMP levels inhibited the release of chemotactic factors into the Arthus-induced pleural effusions. The different effect of elevated cyclic AMP on cell migration in the different types of acute inflammation studied is difficult to explain. It is not due to an inhibition of particle ingestion since other experiments using the same models have shown that dibutyryl cyclic AMP and theophylline have no effect on leucocyte phagocytosis *in vivo* (Deporter *et al.*, 1977). However, the difference may be related to the fact that only the pleurisy induced by immediate hypersensitivity is complement-dependent (Yamamoto *et al.*, 1975). The two types of crystal-induced inflammation are not complement-dependent (Willoughby, Dunn, Yamamoto, Capasso, Deporter & Giroud, 1975; McCarty & Kozin, 1975). Complement generates factors that are chemotactic for PMNs (Ward, 1974) but the effect of cyclic AMP on the generation of these factors is not yet known and requires *in vitro* investigation.

The present results with urate crystals are in disagreement with those of Tse & Andrews (1973) who showed that the release of a chemotactic factor from PMNs following the intra-articular injection of urate crystals in dogs could be inhibited by injecting cyclic AMP with the crystals. The conflicting results could be due to differences in crystal preparation if, for example, Tse & Andrews did not include heat sterilization at 180°C. Non-heated urate crystals are known to activate the complement system (McCarty & Kozin, 1975). Also, since cyclic AMP levels in the dog leucocytes were not monitored, the inhibition of chemotaxis observed may not have been associated with elevated leucocyte cyclic AMP content. Cyclic AMP penetrates cell membranes less effectively than its dibutyryl derivative and is rapidly degraded by cyclic AMP phosphodiesterase (Robison, Butcher & Sutherland, 1971). Furthermore, *in vitro* butyrylated cyclic AMP derivatives and theophylline but not exogenous cyclic AMP mimic the effects of increased intracellular cyclic AMP (Drezner, Neelson & Lebovitz, 1976).

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# THE POSSIBLE ROLES OF HISTAMINE, 5-HYDROXYTRYPTAMINE AND PROSTAGLANDIN $F_{2\alpha}$ AS MEDIATORS OF THE ACUTE PULMONARY EFFECTS OF ENDOTOXIN

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1 In an attempt to investigate the possible role of released vasoactive substances in mediating the pulmonary pressor responses to *E. coli* endotoxin, cats were pretreated with histamine, 5-hydroxytryptamine (5-HT) or prostaglandin antagonists, with a histamine depleting agent (compound 48/80) or with an inhibitor of prostaglandin synthetase (sodium meclofenamate).

2 The administration of endotoxin (2 mg/kg) resulted in a rapidly developing pulmonary hypertension (pressure twice normal after 2–3 min), increases in right atrial and intratracheal pressures, systemic hypotension and bradycardia. These effects were unaffected by methysergide in a dose sufficient to prevent the effects of intravenously administered 5-HT.

3 Endotoxin responses were also unaffected by a combination of mepyramine and burimamide in doses sufficient to reduce markedly the effects of intravenously-administered histamine. In cats pretreated (chronically or acutely) with compound 48/80, endotoxin induced a transient pulmonary pressor response which was not maintained.

4 The pulmonary and systemic responses to endotoxin were prevented by the prior administration of the prostaglandin antagonist, polyphloretin phosphate and by pretreatment with the prostaglandin synthetase inhibitor, sodium meclofenamate.

5 It is concluded that a pulmonary vasoconstrictor prostaglandin is involved in the acute response to endotoxin in the cat.

## Introduction

A large number of vasoactive agents are released, in a number of species, following the intravenous administration of endotoxin. These include histamine (Weil & Spink, 1957; Hinshaw, Jordan & Vick, 1961; Hinshaw, 1964), 5-hydroxytryptamine (5-HT) (Armin & Grant, 1957; Davies, McQuarrie & Meeker, 1959), angiotensin (Hall & Hodge, 1971), adrenaline and/or noradrenaline (Nykiel & Glaviano, 1961; Hökfelt, Bygdeman & Sekkenes, 1962; Hall & Hodge, 1971) and 'a cholinergic substance' (Vick, 1965). There is also early activation of the kallikrein-kinin system (Erdös & Miwa, 1968; Nies, Forsyth, Williams & Melmon, 1968; Al-Kaisi, Parratt, Siddiqui & Zeitlin, 1976). More recently, there has been increasing evidence, in a number of species for the release by endotoxin of both E and F prostaglandins (Collier, Herman & Vane, 1973; Kessler, Hughes, Bennett & Nadela, 1973; Herman & Vane, 1974; 1976; Anderson, Jubiz, Tsagaris & Kuida, 1975a; Anderson, Tsagaris, Jubiz & Kuida, 1975b; Herman & Moncada, 1975; Korbut, Ocetkiewicz & Gryglewski, 1975).

The relevance of the release of so many highly active substances to the pathophysiology of endotoxin shock is still not clear despite attempts (Vick, 1960; 1965; Vick, Mehlman & Heiffer, 1971) to correlate early cardiovascular changes with the release of one or other of these agents. There are at least two possible approaches to the problem: (1) the detection of vasoactive agents in blood following endotoxin administration; (2) the classical pharmacological approach, of prevention of synthesis or release, and antagonism of effects of released vasoactive agents. The latter is the approach used in the present experiments. Particular attention has been paid to the initial pulmonary effects of endotoxin in the cat (hypertension, oedema and the reduction in pulmonary compliance) because of the possible relevance to the marked pulmonary changes that occur in patients with septic shock (Pontoppidan, Geffin & Laver, 1971), a condition in which circulating endotoxin levels are often high (Ledingham & McCartney, personal communication).

Three possible vasoactive agents have been examined, histamine, 5-HT and a prostaglandin of the F series. Each has pulmonary effects similar to endotoxin and there is evidence that each is released, at least in some species, following endotoxin administration. A preliminary account of some of the experiments described in this paper was given to a meeting of the Physiological Society (Parratt & Sturgess, 1975a).

## Methods

Cats, of either sex, were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (35 mg/kg), were subjected to thoracotomy and ventilated with room air by means of a Palmer positive-pressure pump. Systemic (carotid) arterial pressure and d*P*/d*t*, pulmonary arterial pressure, right atrial pressure, cardiac output and stroke volume, systolic ejection time, intratracheal pressure, arterial blood gases and pH and arterial lactate and glucose concentrations were measured as described previously (Parratt, 1973; Parratt & Sturgess, 1976).

*Escherichia coli* endotoxin (Difco Laboratories 055:B5) in a dose of 2 mg/kg, suspended in 0.9% w/v NaCl solution (saline), was injected slowly, over a period of 40–45 s, into a catheter inserted in a femoral vein. Twenty-three cats were given endotoxin only and served as the control group. Particular attention was paid to the initial haemodynamic response and a continuous record was obtained of effects occurring in the first 10 min; thereafter recordings were made every 5 min up to 1 h and then every 30 min until the animal died.

### *Experiments with antagonists of vasoactive agents released by endotoxin*

The following drugs were administered intravenously prior to endotoxin: (1) A combination of mepyramine (2 mg/kg) and burimamide (2 mg/kg), to antagonize the effects of released histamine (6 cats); or (2) methysergide (1 mg/kg), to antagonize the effects of released 5-HT (6 cats) or (3) polyphloretin phosphate, in doses up to 200 mg/kg to antagonize some of the cardiovascular effects of released prostaglandins (6 cats).

Polyphloretin phosphate (a generous gift from Dr B. Hogberg of A.B. Leo, Halsingborg, Sweden) was dissolved in 0.1 N NaOH and injected slowly intravenously over 5 to 10 minutes.

### *Experiments with compound 48/80*

Two groups of cats were administered compound 48/80: (i) the drug was administered acutely to 7 anaesthetized cats in gradually increasing doses (1, 2, 5, 10, 20 and 50 μg/kg by intravenous injection) until the typical haemodynamic responses (hypotension, and increases in pulmonary artery pressure, heart rate and carotid d*P*/d*t* *max;* Table 4) were abolished or markedly attenuated (i.e. until the animals became refractory to further injections of the drug); (ii) four cats were treated with compound 48/80, by intraperitoneal injection over four days, according to the schedule described by Waton & West (1966). This procedure reduced abdominal skin (mast cell) histamine by about 90% (see Results section) but had little effect on the histamine content of the intestine or lung.

Twenty-four hours after the last injection the cats were anaesthetized and prepared as described above. Biopsies taken from the skin of the abdomen, the lung and from the first part of the jejunum, were extracted and assayed for histamine as outlined by Waton & West (1966). At the end of the experiments (1 to 2 h after the administration of endotoxin) small pieces of abdominal skin, lung and small intestine were again removed and their histamine content determined, in order to see whether endotoxin itself released histamine.

### *Experiments with sodium meclofenamate*

Sodium meclofenamate was given to 7 cats, in an intravenous dose of 2 mg/kg, 30 min before the intravenous administration of endotoxin. This dose was chosen because in preliminary experiments it had been found (Parratt & Sturgess, 1975b) to abolish the initial effects of endotoxin even when given up to 5 h beforehand.

## Results

### *Haemodynamic effects of* E. coli *endotoxin*

The intravenous administration of endotoxin resulted in marked haemodynamic changes within 1 min of the injection and indeed, often whilst the injection was still in progress. The main effects were increases in pulmonary artery and intratracheal pressures, a transient bradycardia and a systemic hypotension. The results from the 23 cats are summarized in Table 1. They were compared with those obtained when the same dose (and batch) of endotoxin was administered to cats pretreated with drugs which would either block the effects of released vasoactive substances or which would interfere with their storage or synthesis.

### *The possible role of released histamine*

(a) *Evidence from experiments using a combination of* $H_1$ *and* $H_2$*-receptor blocking agents:*

(i) *Responses to histamine infusions, and to injections of compound 48/80.* Black, Owen & Parsons (1975), Owen (1975) and Flynn & Owen (1975), have clearly demonstrated that histamine-induced vasodepression is only partially inhibited by $H_1$ and $H_2$-receptor antagonists given separately. We therefore gave a combination of mepyramine and burimamide. When given together, these drugs had no significant effect on diastolic pressure (74 ± 8 mmHg before the injection and 76 ± 11 mmHg 1 min afterwards; 1 mmHg ≃133Pa), on mean pulmonary artery pressure (12.4 ± 0.5 and 12.4 ± 1.1 mmHg) or on systolic ejection time (118 ± 6 and 127 ± 7 milliseconds). Heart rate was slightly, although not significantly, reduced (from 226 ± 14 to 192 ± 15 beats/minute). The haemodynamic responses to intravenous infusions of histamine (5 μg $kg^{-1}$ $min^{-1}$) were almost completely abolished by this combination of antagonists (Table 2). The haemodynamic effects of released histamine were also markedly reduced. For example, compound 48/80 (10 μg/kg) lowered diastolic blood pressure by a mean of 34 mmHg before the administration of the two blocking drugs and by only 7 mmHg afterwards; the corresponding figures for carotid d*P*/d*t* *max* were $^{+}$578 $mmHgs^{-1}$ and $^{+}$139 $mmHgs^{-1}$ and, for pulmonary artery pressure, $^{+}$1.8 mmHg and 0.3 mmHg. We expected on the basis of these results that this combination of antagonists would greatly reduce responses to *E. coli* endotoxin if these were in fact due to histamine release.

(ii) *Responses to* E. coli *endotoxin.* The initial pulmonary hypertension and systemic vasodepression that resulted from the administration of endotoxin were in no way modified by the combination of mepyramine and burimamide (Table 3) and the time course of the effects was similar to that found in cats administered endotoxin alone (Figure 1). For example, the systemic and pulmonary pressures 1 h after endotoxin in the cats pretreated with mepyramine and burimamide (74 ± 4 mmHg and 13 ± 1.2 mmHg respectively) were similar to those obtained following endotoxin in the control group of cats (75 ± 8 mmHg and 15.6 ± 2.0 mmHg). The 'delayed' effects of endotoxin (reduced cardiac output, lactate production, hypoglycaemia) were also apparent in the cats treated with the blocking drugs. Thus after 3 h the cardiac output was reduced from 119 ± 16 to 70 ± 8 ml $min^{-1}$ $kg^{-1}$ ($P<0.05$), the stroke volume from 1.61 ± 0.24 to 0.93 ± 0.18 ml/beat, and the arterial glucose from 144 ± 28 to 66 ± 25 mg/100 ml; arterial lactate had risen from 11.7 ± 2.7 to 30 ± 6.8 mg/100 ml. These changes were similar to those observed in cats administered *E. coli* endotoxin alone. However, there was an indication that survival rate was increased by the combination of mepyramine and burimamide; 2 of the 6 cats were alive at 6 h compared to 2 out of the 23 administered endotoxin only.

**Table 1** The acute haemodynamic effects of *E. coli* endotoxin in anaesthetized cats

| | *Time after endotoxin administration (min)* | | | | | |
|---|---|---|---|---|---|---|
| | *0* | *1* | *3* | *4* | *5* | *10* |
| Carotid artery pressure: | | | | | | |
| systolic (mmHg) | 110 ± 5 (23) | 73 ± 10 (17)* | 98 ± 10 (17) | 110 ± 11 (15) | 112 ± 8 (22) | 102 ± 5 (22) |
| diastolic | 83 ± 5 (23) | 50 ± 8 (17)** | 61 ± 9 (17)** | 73 ± 8 (15) | 77 ± 6 (22) | 71 ± 6 (22) |
| Pulmonary artery pressure: | | | | | | |
| systolic (mmHg) | 18.8 ± 0.9 (12) | 26.1 ± 2.2 (11)* | 36.9 ± 3.8 (10)** | 41.3 ± 4.5 (8)** | 36.3 ± 4.5 (8)** | 30.8 ± 2.6 (10) |
| diastolic (mmHg) | 9.4 ± 0.4 (12) | 17.8 ± 2.4 (11)* | 23.2 ± 2.7 (10)** | 26.0 ± 2.9 (8)** | 23.1 ± 2.1 (12)** | 18.6 ± 1.7 (10) |
| Right atrial pressure (mean, mmHg) | 0.9 ± 0.3 (11) | 1.7 ± 0.2 (5)** | 2.7 ± 1.3 (5) | 2.7 ± 1.2 (8) | 1.2 ± 1.1 (8) | – |
| Heart rate (beats/min) | 196 ± 8 (23) | 174 ± 15 (17) | 160 ± 16 (17)** | 199 ± 17 (14) | 196 ± 11 (20) | 201 ± 9 (22) |
| Intratracheal pressure (% pre-endotoxin level) | 100 (5) | 191 ± 26 (5)** | 180 ± 20 (5)** | 172 ± 21 (5)** | 164 ± 9 (5)* | 174 ± 12 (5)* |

Values are mean ± s.e. mean Number of experiments in parentheses
* $P<0.001$, ** $P<0.01$

### (b) *Evidence from the use of compound 48/80*

(i) *Results from cats given cumulative single injections.* Compound 48/80 was given in increasing doses by intravenous injection until the haemodynamic effects were abolished or markedly attenuated. Small doses (1–5 μg/kg) usually slightly elevated both systemic and pulmonary arterial pressures; higher doses (10–50 μg/kg) reduced systemic arterial pressure but still induced pulmonary hypertension (Table 4). Carotid d*P*/d*t max* and heart rate were always increased. The final effects of the cumulative intravenous administration of the drug were a significantly reduced systemic arterial pressure (Table 4), a reduced cardiac output (from 244 ± 17 to 127 ± 11 ml/min) and stroke volume (from 2.8 ± 0.4 to 1.8 ± 0.15 ml/beat); there were increases in arterial lactate (from 7.0 ± 1.3 to 32.6 ± 7.9 mg/100 ml) and glucose (from 106 ± 23 to 224 ± 67 mg/100 ml).

The effect of administering endotoxin to these cats is shown in Figure 2. Systemic hypotension and bradycardia still occurred and, in the first few minutes, there was an elevation of pulmonary artery pressure (from 13.0 ± 1.4 mmHg to 22.4 ± 4.8 mmHg after 1 min, 19.8 ± 5.0 mmHg after 2 min and 18.6 ± 4.9 mmHg after 3 minutes). However, the pulmonary artery pressure 5 min after endotoxin (13.9 ± 2.0 mmHg) was not significantly different from the pre-endotoxin level. The transient nature of this pulmonary hypertensive response was in contrast to the response in the control animals (Table 1). All the cats pretreated with compound 48/80 exhibited a raised arterial lactate 1 h after endotoxin (48.1 ± 10.3 mg/100 ml) and none of the 6 animals were alive at 4 hours.

(ii) *Results from cats pretreated chronically with compound 48/80.* Only 3 of the 4 cats survived the dosage schedule outlined in the Methods section. Biopsies taken from 3 of the animals before the administration of endotoxin showed that skin histamine was greatly depleted (values of 0.47, 0.23 and 1.25 μg/g). The histamine content of the lung (24,

**Table 2** Haemodynamic effects of intravenous infusions of histamine (5 μg $kg^{-1}$ $min^{-1}$) before, and after, the administration of mepyramine (2 mg/kg) plus burimamide (2 mg/kg)

| | *Before* | | *After* | |
|---|---|---|---|---|
| | *Control* | *Change* | *Control* | *Change* |
| Carotid artery pressure: | | | | |
| systolic (mmHg) | 102 ± 11 | −24 ± 5 (6) | 103 ± 8 | −3 ± 1 (6)* |
| diastolic (mmHg) | 75 ± 10 | −37 ± 8 (6) | 71 ± 8 | −5 ± 1 (6)* |
| Pulmonary artery pressure: | | | | |
| systolic (mmHg) | 18.1 ± 0 8 | +3 7 ± 1 5 (6) | 19.0 ± 1 0 | +0.8 ± 0.2 (6)** |
| diastolic (mmHg) | 9.5 ± 0.4 | +1.0 ± 0.5 (6) | 9.7 ± 0.4 | +0.2 ± 0.2 (6)† |
| Heart rate (beats/min) | 219 ± 17 | +21 ± 6 (6) | 206 ± 13 | +2 ± 5 (6)** |

$^{*}P < 0.001$; $^{**}P < 0.02$, $^{\dagger}P < 0.05$ compared with change induced before the histamine antagonists

**Table 3** The acute haemodynamic effects of endotoxin in anaesthetized cats when administered after a combination of mepyramine (2 mg/kg) plus burimamide (2 mg/kg)

| | *Time after endotoxin administration (min)* | | | | | |
|---|---|---|---|---|---|---|
| | *0* | *1* | *3* | *4* | *5* | *10* |
| Carotid artery pressure: | | | | | | |
| systolic (mmHg) | 98 ± 7 | 73 ± 16 | 91 ± 12 | 113 ± 13 | 106 ± 9 | 90 ± 6 |
| diastolic (mmHg) | 66 ± 6 | 46 ± 14 | 53 ± 13 | 74 ± 13 | 70 ± 10 | 53 ± 4 |
| Pulmonary artery pressure: | | | | | | |
| systolic (mmHg) | 18 8 ± 1.3 | 32.3 ± 3.0** | 36.2 ± 3.9** | 32.2 ± 3.3** | 30 6 ± 3.4** | 29 7 ± 4.2† |
| diastolic (mmHg) | 9.2 ± 0.4 | 23.4 ± 3.3** | 23.5 ± 2.3* | 21.4 ± 2.7* | 20.6 ± 3 2** | 18.0 ± 2.8† |
| Heart rate (beats/min) | 215 ± 15 | 175 ± 25 | 201 ± 12 | 236 ± 30 | 226 ± 26 | 223 ± 26 |

Results are mean of 6 experiments ± s.e. mean.
$^{*}P < 0.001$; $^{**}P < 0.01$; $^{\dagger}P < 0.02$.

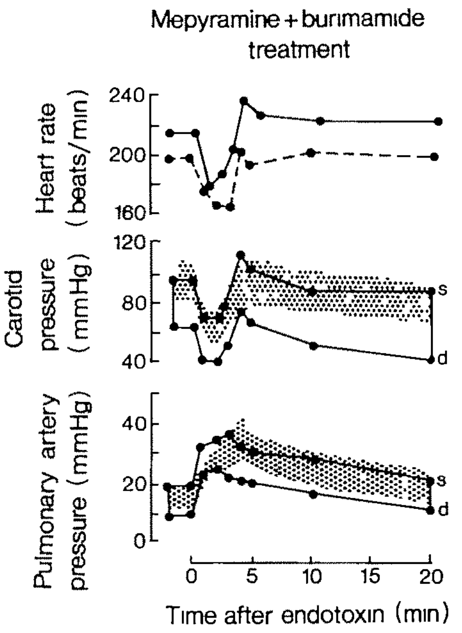

**Figure 1** The effects of *E. coli* endotoxin (2 mg/kg; at time zero) on heart rate, carotid artery and pulmonary artery pressures (s = systolic; d = diastolic) when administered after a combination of mepyramine and burimamide (2 mg/kg of each; ●——●) The values are the means obtained from 6 animals; standard errors have been omitted for clarity. The shaded areas and points joined with broken lines (●– –●) in this and in Figures 2–6 represent the effects of endotoxin in 11–22 control (untreated) cats. It is clear that a combination of $H_1$ and $H_2$-receptor blocking agents does not modify the haemodynamic effects of endotoxin.

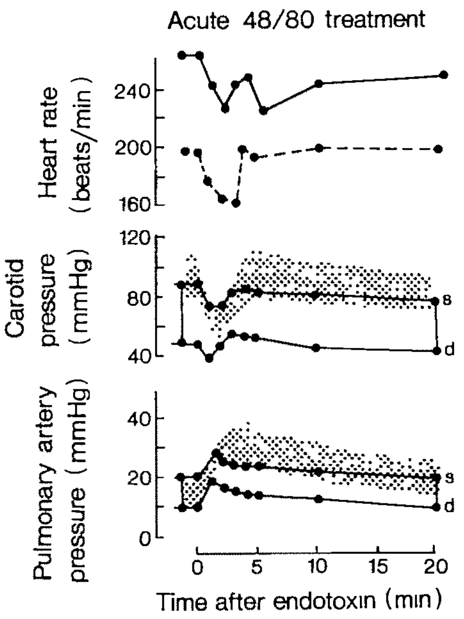

**Figure 2** The haemodynamic effects of endotoxin (2 mg/kg at time zero) in cats treated acutely with compound 48/80 to deplete mast cells of histamine (●——●). The values are the means obtained from 7 experiments, standard errors have been omitted for clarity The effects of endotoxin are reduced and the rise in pulmonary artery pressure is not sustained compared with control cats administered endotoxin (shaded areas).

**Table 4** Haemodynamic responses to cumulative single intravenous injections of compound 48/80

| | | *Dose of 48/80* *(Change from pre-injection level*)* | | | |
|---|---|---|---|---|---|
| | *Control* | *5 µg/kg* | *10 µg/kg* | *20 µg/kg* | *Post-cumulative administration†* |
| Carotid artery pressure: | | | | | |
| systolic (mmHg) | 130 ± 8 | +5 ± 3 | −8 ± 5 | −16 ± 7 | 89 ± 6** |
| diatolic (mmHg) | 99 ± 7 | +5 ± 3 | −18 ± 8 | −31 ± 11 | 48 ± 8** |
| Pulmonary artery pressure: | | | | | |
| systolic (mmHg) | 23.7 ± 2.2 | +1.5 ± 0.7 | +3.8 ± 1 4 | +5.2 ± 2.0 | 21.0 ± 1.6 |
| diastolic (mmHg) | 13.0 ± 1.4 | +0.8 ± 0.3 | +1.1 ± 0.5 | +1.5 ± 0 6 | 10.3 ± 1.5 |
| Heart rate /beats/min) | 228 ± 19 | +5 ± 4 | +14 ± 5 | +23 ± 12 | 264 ± 13 |

Results are means of 7 experiments ± s.e. mean

* Apart from the systemic arterial and heart rate responses to a dose of 5 µg/kg all of the changes induced by compound 48/80 were significantly ($P < 0.001$) different from those induced by an equal volume of saline.

† Values are those obtained 30–40 min after the cumulative administration of 200 µg/kg compound 48/80 and immediately before administration of endotoxin. ** $P < 0.01$

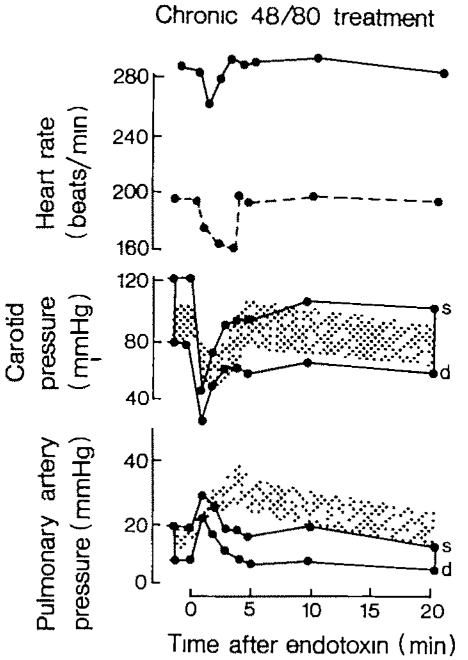

**Figure 3** The haemodynamic effects of endotoxin (2 mg/kg at time zero) in 3 cats treated over 4 days with compound 48/80 in order to deplete mast cells of histamine (●—●). The systemic hypotensive effect of endotoxin is unaffected by this treatment but the marked elevation of pulmonary artery pressure observed in control cats (shaded areas) is not sustained.

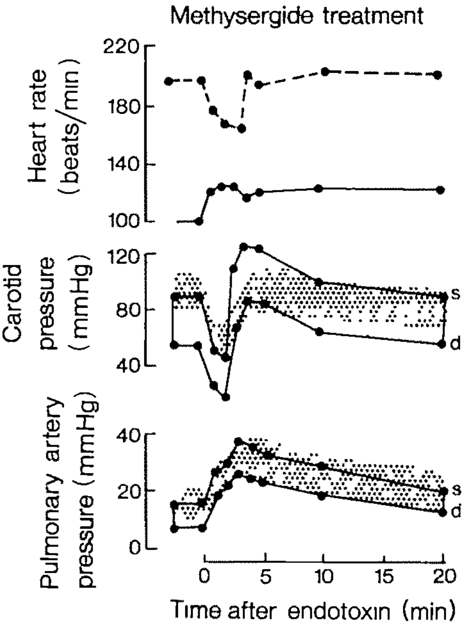

**Figure 4** The haemodynamic effects of endotoxin (2 mg/kg at time zero) in 3 cats administered methysergide in a dose (1 mg/kg) that prevented the effects of 5-hydroxytryptamine. The vascular effects of endotoxin were unaffected by this treatment; the bradycardia was abolished (●—●).

23 and 22.5 μg/g) and jejunum (73.8, 71.3 and 45.7 μg/g) were in the normal range for this species (Smith, 1953). These results are in accord with the view that compound 48/80 depletes histamine only from those tissues where it resides in mast cells (Parratt & West, 1957).

The effects of endotoxin in these 'mast cell histamine depleted cats' are illustrated in Figure 3. They were basically similar to those obtained in cats treated acutely with compound 48/80 (Figure 2). There was again a transient pulmonary hypertension (from 13.5 to 26.9 mmHg after 1 min, 21.9 mmHg after 2 min and 16.5 mmHg after 3 minutes). Once again, the pressure after 5 min (12.6 mmHg) was not significantly different from the pre-endotoxin level.

None of these animals survived 3 h and, before death, they all had raised arterial lactate levels and a reduced cardiac output. Samples of intestine were taken at autopsy and there was some evidence that the histamine levels were reduced (46.8 and 58.8 μg/g respectively compared to the pre-endotoxin levels in these same animals of 73.9 and 71.3 μg/gram). In contrast, skin histamine was not further reduced (values of 0.78, 0.78 and 1.26 μg/gram).

### *The possible role of 5-hydroxytryptamine*

Intravenous injections of 5-HT (10, 20 and 40 μg/kg) reduced systemic (carotid) arterial pressure (by 29 ± 9 mmHg with a dose of 10 μg/kg, 36 ± 12 mmHg with 20 μg/kg and by 54 mmHg after a dose of 40 μg/kg, from the pre-injection diastolic pressure of 95 ± 9 mmHg) and heart rate (−66 ± 7 beats/min after 20 μg/kg and −111 ± 19 beats/min after 40 μg/kg; control level 224 ± 16 beats/minute). There were also increases in pulmonary artery pressure, although these were not dose-related (increases of 3.5 ± 2.0 and 2.9 ± 3.3 mmHg from the control level of 10.1 ± 1.3 mmHg after 10 and 20 μg/kg respectively). Intratracheal pressure was also increased (by 0.3 ± 0.2, 0.7 ± 0.2 and 1.2 mmHg respectively from the resting peak inspiratory pressure of 7.7 ± 0.6 mmHg). These effects were greatly reduced by the prior administration of methysergide (1.0 mg/kg). For example, following a dose of 20 μg/kg of 5-HT, diastolic pressure was reduced by only 6 mmHg (compared to 36 mmHg) heart rate by 18 beats/min (compared to 66 beats/min) and the rise in pulmonary artery pressure which occurred before methysergide was converted to a slight fall (of 2 mmHg).

Unfortunately methysergide itself had marked effects on the cardiovascular system. There were reductions in systemic pressure (by a mean of 40 mmHg diastolic and 37 mmHg systolic) and in heart rate (63 beats/min), effects rather similar to those of 5-HT itself. Despite these intrinsic effects it was clear that this dose of methysergide greatly modified the responses to injected 5-HT and therefore could be used to examine the possible contribution of released 5-HT to the acute haemodynamic effects of endotoxin. Figure 4 shows that the cardiovascular effects of endotoxin were quite uninfluenced by methysergide.

### *The possible role of a released (pulmonary vasoconstrictor) prostaglandin*

Two possible approaches were investigated; prevention of the effects of released prostaglandins (with polyphloretin phosphate) and prevention of any possible release by inhibition of prostaglandin synthetase (with sodium meclofenamate; Flower, 1974).

(a) *Evidence from the effects of polyphloretin phosphate.* Polyphloretin has been shown to reduce the effects of prostaglandin $F_{2\alpha}$ in cats (Villanueva, Hinds, Katz & Eakins, 1972). Unfortunately, although certainly effective in reducing the pulmonary effects of injected or infused prostaglandin $F_{2\alpha}$, it had considerable inherent depressive effects on the cardiovascular system. Systolic and diastolic carotid pressures were reduced from 128 ± 12/100 ± 10 mmHg (mean pressure 117 ± 11 mmHg) to 67 ± 10/35 ± 12 mmHg (mean pressure 45 ± 11 mmHg; $P < 0.001$), mean pulmonary artery pressure was reduced from 16.5 ± 1.2 mmHg to 11.3 ± 1.5 mmHg ($P < 0.05$), heart rate from 246 ± 10 to 213 ± 24 beats/min ($P > 0.05$) and cardiac output from 373 ± 52 to 110 ± 45 ml/min ($P < 0.01$). Peripheral vascular resistance was unchanged.

After 1 h there was some recovery (carotid artery pressure of 82 ± 12/50 ± 10 mmHg) and at this time the pulmonary hypertensive effects of injected prostaglandin $F_{2\alpha}$ (though not the effects on intratracheal pressure) - were greatly reduced (Table 5). There appeared to be some selectively of action because, in three experiments, the hypotensive effect of intravenously administered histamine (1.5 μg/kg) was only slightly reduced by polyphloretin.

The effects of endotoxin were completely prevented by polyphloretin (Figure 5); there was no reduction in carotid artery pressure, no increase in pulmonary artery pressure and only a slight (14–22%) increase in intratracheal pressure.

(b) *Evidence from the administration of sodium meclofenamate.* In doses up to 2 mg/kg (14 experiments), sodium meclofenamate had no effect on systemic or pulmonary artery pressures, on heart rate, systolic ejection time, cardiac output, peripheral vascular resistance or on arterial blood $P\text{O}_2$, $P\text{CO}_2$, pH, lactate or glucose. Detailed results are available from the authors.

When endotoxin was injected 30 min after sodium meclofenamate the initial effects on carotid and pulmonary artery pressures were considerably (and significantly) reduced. This is clear from Figure 6. There was only a slight, transient, pulmonary hypertensive effect in the first 2 min at the injection (an increase in mean pulmonary artery pressure from 15.8 ± 1.0 mmHg before endotoxin to 19.5 ± 1.0 mmHg 1 min afterwards ($P < 0.01$) and to 19.2 ± 0.9 mmHg after 2 min; $P < 0.05$). This effect was in no way comparable to that seen in the cats administered endotoxin only (Table 1 and Figure 6).

**Table 5** Pulmonary arterial pressure responses to injections of prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$) before, and after, the administration of polyphloretin phosphate (200 mg/kg)

| | | *Change in pressure induced by PG $F_{2\alpha}$ (μg/kg) before (b) and after (a) polyphloretin* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *1.25* | | *2.5* | | *5.0* | |
| | *Control* | *b* | *a* | *b* | *a* | *b* | *a* |
| Pulmonary artery pressure· | | | | | | | |
| systolic (mmHg) | 21.8 ± 1.8 | +10.3 ± 2.5 | +3.4 ± 1.5** | +17.2 ± 2.4 | +4.9 ± 0.6* | +24.5 ± 1.4 | +6.7 ± 2.0* |
| diastolic (mmHg) | 10.6 ± 0.6 | +6.6 ± 1.6 | +2.9 ± 1.0** | +10.0 ± 1.6 | +4.1 ± 0.5* | +15.3 ± 1.6 | +5.6 ± 1.5* |

Results are means of 6 experiments ± s.e. mean.
Change after polyphloretin significantly different from change before polyphloretin (*$P < 0.001$; ** $P < 0.01$)

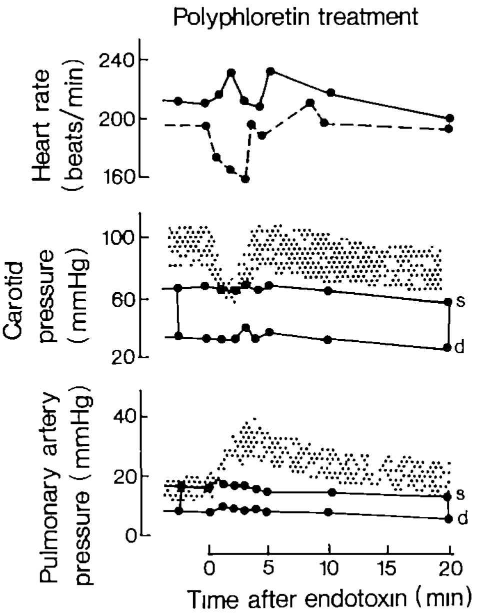

**Figure 5** The haemodynamic effects of endotoxin (2 mg/kg at time zero) in 6 cats pretreated with polyphloretin phosphate (200 mg/kg; ●——●). There is an almost complete abolition of the effects of endotoxin normally observed in untreated animals (shaded areas).

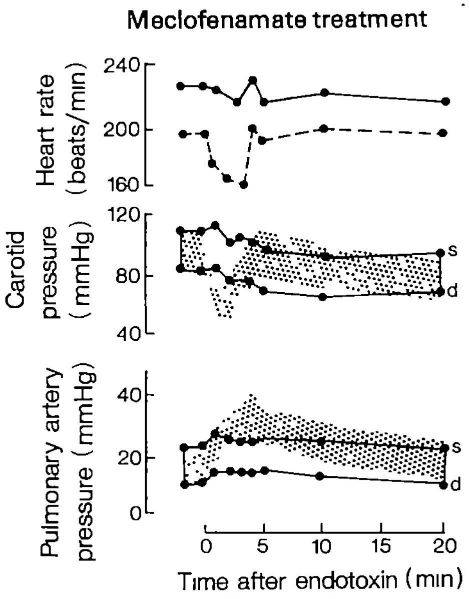

**Figure 6** The haemodynamic effects of endotoxin (2 mg/kg at time zero) in 7 cats pretreated with sodium meclofenamate (2 mg/kg, ●——●). The change in systemic and pulmonary arterial pressures (shaded areas) and in heart rate (●– – –●) seen in untreated cats are not observed in cats pretreated with sodium meclofenamate.

There was no acute systemic hypotensive effect (Figure 6) although pressure tended to decrease slightly (by 15–19 mmHg), although not significantly, after 5–10 minutes. The endotoxin-induced bradycardia was absent in cats treated with meclofenamate.

We have not described the results obtained when endotoxin was administered at times other than 30 min after sodium meclofenamate. The acute pulmonary effects of endotoxin were greatly reduced, even when administered up to 5 h after meclofenamate (Sturgess, 1975).

## Discussion

One of the principal aims of the present study was to examine which, if any, of the humoral vasoactive mediators released by endotoxin is primarily responsible for the marked pulmonary changes that result from the acute administration of lipopolysaccharide. This may have some relevance to the 'shock lung' syndrome commonly observed in patients with septic shock (Wilson, 1972). These pulmonary changes include oedema, atelectasis, a reduced arterial oxygen tension (often below 60 mmHg), hyperventilation and an abnormal alveolar-arterial oxygen tension gradient (Pontoppidan *et al.*, 1971; Milligan, MacDonald, Mellon & Ledingham, 1974). The mechanisms producing this pulmonary lesion are not known; the most likely appear to be sequestration of platelet aggregates and the release of, as yet unidentified, vasoactive agents (Milligan *et al.*, 1974).

The administration of purified *E. coli* endotoxin in the cat results in pulmonary hypertension, oedema, a reduced arterial oxygen tension and a decreased airways compliance (Kuida, Hinshaw, Gilbert & Visscher, 1958; Kuida, Gilbert, Hinshaw, Brunson & Visscher, 1961; Parratt, 1973; Parratt & Sturgess, 1976). This response begins within 1 min of endotoxin administration and pulmonary artery pressure is more than twice normal 3 to 4 min after the injection (Table 1). There are three possible explanations for this increase in pressure, a direct vasoconstrictor effect of endotoxin on pulmonary vascular smooth muscle, mechanical blockage (by particulate endotoxin or clumped blood cells) or the release of some humoral pulmonary broncho- and vasoconstrictor. The first possibility is unlikely since addition of an endotoxin suspension to an organ bath containing pulmonary arterial or venous smooth muscle strips does not result in contraction (Parratt & Sturgess, unpublished observations). Mechanical obstruction is more likely, particularly since endotoxin induces platelet clumping and thrombocytopenia (Kitzmiller, Lucas & Yelenosky, 1972; Robb, Margulis & Jabs, 1972; Lucas & Kitzmiller, 1972). However there are a number of observations which argue against small vessel obstruction *per se* being the most significant mechanism: (i) The reduction in platelet count in the present experiments, at the height of the pulmonary pressor response, was not dramatic. Thus 5 min after endotoxin the platelet count had fallen from $324 \pm 91 \times 10^3$ per mm$^3$ to $268 \pm 77 \times 10^3$ per mm$^3$ ($P < 0.05$). (ii) Inhibition of platelet aggregation with carbochromen and with dipyridamole does not influence pulmonary hypertension induced by endotoxin (Parratt & Sturgess, 1975a). (iii) In complement-depleted cats the thrombocytopenic response to endotoxin is much reduced, although the pulmonary lesions in these animals (congestion and oedema) are no different from those found in normal cats given endotoxin (Kitzmiller *et al.*, 1972). (iv) Sheep, like cats, respond to endotoxin by a marked increase in pulmonary artery pressure. In pairs of anaesthetized sheep with a connecting unilateral carotid-jugular anastomosis, pulmonary embolism in the donor sheep produces an increase in pulmonary artery pressure (and a reduction in airways compliance) in the recipient animal (Halmagyi, Starzecki & Horner, 1964). This suggests that humoral factors predominate. (v) Protamine chloride, like endotoxin, causes a marked thrombocytopenia (due to platelet aggregation) and pulmonary hypertension when infused into heparinized animals. The pressor response, but not the platelet aggregation, is inhibited by aspirin (Radegran & McAslan, 1972). This again suggests that the release of a smooth muscle contracting substance, and not mechanical (intravascular) obstruction, is the predominant mechanism involved.

The present experiments allow only tentative conclusions to be drawn as to which humoral substance is primarily responsible for mediating the pulmonary responses induced by endotoxin. It is unlikely that 5-HT is involved to any great extent since the endotoxin effects on pulmonary and systemic arterial pressure were unaffected by methysergide in a dose that completely prevented the effects of injected 5-HT (Figure 4). The experiments with the antagonists mepyramine and burimamide suggest that histamine is of relatively minor importance (Figure 1); we have additional evidence that even in huge doses (up to 70 mg/kg) mepyramine alone does not inhibit the pulmonary pressor response to endotoxin (Sturgess, 1975). On the other hand, the experiments with compound 48/80 might suggest that histamine is involved in maintaining, but not initiating, the pulmonary response. The increase in pulmonary artery pressure at 1 min was as marked in the compound 48/80-treated cats as in the control (endotoxin alone) cats; however at 2 min (and thereafter) the response was significantly ($P < 0.01$) less than in the cats administered only endotoxin (Figures 2 and 3). Previous studies (Hinshaw, Emerson, Iampietro & Brake, 1962; Parratt, 1973) have also demonstrated that some of the haemodynamic effects of endotoxin (elevation of portal venous pressure in the dog and of pulmonary pressure in the cat) are greatly attenuated by compound 48/80. Clearly histamine release does occur after endotoxin administration. This release is not primarily from mast cells (Sandusky, Johnson & Moran, 1973). From the present studies it appears that some at least of the released histamine is derived from sites other than mast cells in the gut.

One explanation for the apparent discrepancy between the results obtained with histamine antagonists and with the histamine releaser is that compound 48/80 may have effects other than mast cell disruption. For example Dawson, Delano, Hamilton & Stekiel (1974), having summarized the considerable evidence that histamine is not the main mediator of hypoxia-induced vasoconstriction in cat isolated lungs, concluded that compound 48/80 inhibits this vasoconstriction by 'a mechanism that does not involve histamine release'. The fact that in the present experiments, the endotoxin response was rather less when compound 48/80 was given acutely (Figure 2) than when given by chronic administration (Figure 3) might imply that the presence of the drug in the circulation was of greater importance than histamine depletion *per se*. As far as we are aware there have been no studies to examine whether compound 48/80 inhibits prostaglandin synthetase. There is however, some evidence (Anggard & Strandberg, 1971) that it can release prostaglandin $F_{2\alpha}$ from perfused cat paws. An effect on prostaglandin synthesis or release would adequately explain both our results with endotoxin and the results of Dawson *et al.* (1974) described above. There is now some evidence that hypoxia induces release of prostaglandins from the lung (Said, Yoshida, Kitamura & Vreim, 1974). A common mediator for the pulmonary vasoconstriction induced by hypoxia and by endotoxin is suggested by experiments of Grover & Reeves (1974); after endotoxin administration the pulmonary pressor response to hypoxia was abolished or markedly reduced.

Most of the evidence from these studies suggests that a pulmonary vasoconstrictor prostaglandin is involved in the acute response to endotoxin in the cat. The effects of endotoxin were abolished by polyphloretin phosphate, in a dose that inhibited the response to exogenous prostaglandin $F_{2\alpha}$, and were completely prevented by the prior administration of sodium meclofenamate. Similar effects were seen with two other inhibitors of prostaglandin synthetase, indomethacin (Parratt & Sturgess, 1974) and sodium flurbiprofen (Parratt & Sturgess, 1976). Furthermore, prostaglandin $F_{2\alpha}$ is known to be present in the lung (Samuelson, 1964) and analysis of pulmonary venous blood of calves administered endotoxin has demonstrated a two-fold increase in prostaglandin $F_{2\alpha}$ (Anderson *et al.*, 1975b). What is still required is a detailed study of the time course and mechanism of this release and its relationship, if any, to endotoxin-induced pulmonary hypertension, increased airways resistance and decreased airways compliance.

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# DISSIMILARITY BETWEEN THE RESPONSES TO ADENOSINE TRIPHOSPHATE OR ITS RELATED COMPOUNDS AND NON-ADRENERGIC INHIBITORY NERVE STIMULATION IN THE LONGITUDINAL SMOOTH MUSCLE OF PIG STOMACH

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1 Transmural electrical stimulation (TMS) of longitudinal smooth muscle strips taken from the cardiac portion of the pig stomach produced biphasic responses consisting of initial contractions followed by relaxations. The excitatory component was enhanced by neostigmine and abolished by atropine. After atropine treatment, TMS and nicotine or 1,1-dimethyl-4-phenyl-piperazinium, caused a relaxation or a relaxation followed by an after-contraction. All of these responses were abolished or reduced reversibly with tetrodotoxin and cocaine, while hexamethonium only abolished the response to ganglion-stimulating agents.

2 The relaxation caused by TMS reached a maximum amplitude at 5–10 Hz, and was entirely resistant to the effects of $\alpha$- and $\beta$-adrenoceptor blocking agents, or a combination of them, and also to guanethidine. These results strongly suggested that the relaxation was elicited by stimulation of intramural non-adrenergic inhibitory neurones.

3 In the presence of atropine and guanethidine, adenosine triphosphate (ATP, 5–20 $\mu$M) caused only a tonic contraction, and ATP (25–200 $\mu$M) or adenosine diphosphate (25–200 $\mu$M) produced a contractile response or a biphasic one (tonic contraction preceded by a slight relaxation). Adenosine monophosphate and adenosine caused only the tonic contraction over the range of concentrations (25–200 $\mu$M).

4 Stimulation of the intramural inhibitory neurones of the tissue consistently evoked an inhibitory junction potential, which showed a summation during repetitive stimulation. One the other hand, ATP elicited mainly a small depolarization of a few mV.

5 When the desensitization to ATP of the muscle was achieved in the presence of atropine and guanethidine, the relaxation induced by stimulation of the non-adrenergic inhibitory neurones could be evoked without any modification.

6 Dipyridamole neither potentiated the inhibitory responses due to stimulation of the intramural inhibitory neurones nor showed any consistent effect on the ATP-induced response.

7 From these results, it is unlikely that ATP, or any related compound, is the transmitter substance of the intramural inhibitory neurones in the longitudinal smooth muscle of the pig stomach.

## Introduction

Adenosine triphosphate (ATP) or a related compound has been proposed by Burnstock, Campbell, Satchell & Smythe (1970) and Burnstock (1972) as a transmitter substance for the intramural inhibitory non-adrenergic, non-cholinergic neurones of the vertebrate gastrointestinal tract (purinergic nerves hypothesis). A parallelism between the effects of exogenously applied ATP or its analogues and the nerve stimulation was taken as evidence for this hypothesis (Burnstock *et al.*, 1970; Burnstock, Satchell & Smythe, 1972; Satchell, Lynch, Bourke & Burnstock, 1972; Satchell, Burnstock & Dann, 1973; Tomita & Watanabe, 1973). However, it has been reported that ATP cannot mimic the stimulatory effect of intramural inhibitory neurones in some gastrointestinal smooth muscle preparations (Burnstock *et al.*, 1970; MacKay & McKirdy, 1972;

Furness & Costa, 1973; Takewaki, Ohashi & Okada, unpublished results). Thus, further comparative experiments should be carried out to determine whether the stimulatory effects of the inhibitory neurones and ATP or its related compounds are the same in various other smooth muscle preparations.

Recently we found that isolated longitudinal smooth muscle from the pig stomach is useful for the investigation of the inhibitory mechanical responses because the tone of the preparation can be maintained at a high level for long periods without spontaneous fluctuations. The primary purpose of the present experiments was to clarify the intramural innervation of the smooth muscle, with special reference to the nature of the inhibitory neurones. It was found that the smooth muscle was supplied by excitatory cholinergic and inhibitory non-adrenergic, non-cholinergic postganglionic nerve fibres. Therefore, we tried to see whether there was any parallelism between the stimulatory effects of non-adrenergic inhibitory nerves and exogenously applied ATP or its analogues in this preparation. Some of these results were described at the 16th Congress of the Japanese Smooth Muscle Society, Fukuoka, 1974.

## Methods

Experiments were carried out on isolated, longitudinal smooth muscles of the pig (Landrace) stomach. A part of the cardiac region of the stomach wall (5 x 5 cm) was removed from healthy adult pigs of either sex, which had been slaughtered at the local abattoir. The strips were then immersed in a cold saline solution and transported to the laboratory with a delay of approximately 60 minutes. After both the mucosal layer and the circular muscle coat were removed, the longitudinal smooth muscles were cut into segments of about 2.0 cm in length and 0.2 cm in width. A segment was placed vertically in an organ bath containing 5 ml of Krebs-Henseleit solution (mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.5; pH 7.3–7.4) equilibrated with a mixture of 95% $O_2$ and 5% $CO_2$. The solution was kept at 37–38°C and flowed continuously into the bath from a reservoir through polyethylene tubing connected to the bottom of the bath by a micropump (Tokyo Rika Kikai, MP-II). The overflowing fluid was removed by suction, and the volume of the bathing solution was kept constant.

The mechanical activities of the preparations were recorded isometrically with mechano-electronic transducers (Toshiba 5734A or Nihon Kohden, SB-IT) on the ink-writing oscillograph (Nihon Kohden, WI-260). The preparation was loaded with a tension of 2 g and allowed to equilibrate for 60–90 min before starting the experiment.

Transmural electrical stimulation (TMS) to the muscles was applied through two parallel, non-polarizing Ag-AgCl electrodes (5 x 25 mm, separated by 5 mm) placed at either side along the whole length of the preparations. An electronic stimulator (Nihon Kohden, MSE-3R) was used to deliver the rectangular pulses. Trains of pulses were applied for 10–20 s at intervals of not less than 3 min, unless otherwise stated.

In some experiments, changes in the membrane potential due to TMS or to added drugs were observed with the conventional sucrose-gap apparatus, as described by Stämpfli (1954) and Burnstock & Straub (1958). The method of stimulation of the intramural nerves was that described by Ohashi & Ohga (1967).

The drugs were applied as injections or as constant infusions. The final concentrations of drugs are expressed as g/ml or molar concentration (M).

Drugs used were: acetylcholine chloride (ACh), adenosine, adenosine-5′-diphosphate sodium salt (ADP), adenosine-5′-monophosphate sodium salt (AMP), adenosine-5′-triphosphate disodium salt (ATP), adrenaline bitartrate, atropine sulphate, carbamoylcholine hydrochloride (carbachol), cocaine hydrochloride, dibenamine hydrochloride, 1-1-dimethyl-4-phenyl-piperazinium (DMPP), dipyridamole, guanethidine monosulphate, hexamethonium chloride, isoproterenol hydrochloride, neostigmine methylsulphate, nicotine bitartrate, noradrenaline bitartrate, phenoxybenzamine hydrochloride, 5-(3-tert-butylamino-2-hydroxy)propoxy-3,4-dihydrocarbostyril hydrochloride (OPC-1085), tetrodotoxin citrate (TTX), and tyramine hydrochloride.

## Results

The tone of the preparation increased during the equilibrium period of 60–90 min, and reached a steady level (5–10 g) of about 2.5–5 times the initial value and was maintained for several hours. Both the development and maintenance of the tonus were not affected either by atropine (1 μg/ml) or TTX (0.5 μg/ml). The tonus, however, fell to a low level without the calcium and at a low temperature (27–28°C).

### *Frequency-response relationships*

Relationships between stimulation frequency (1–80 Hz) and responses were investigated by applying stimuli for 10 s with a fixed pulse of 1.0 ms duration and a supramaximal voltage. TMS caused only contractile responses over the range of frequencies (5–80 Hz) in the low tone preparations ($n = 4$). The contractile response appeared at 5 Hz and reached a maximum at 40–80 Hz. In contrast, in the tonic preparations (Figure 1a), only a relaxation was obtained at a low range of frequencies (1–5 Hz,

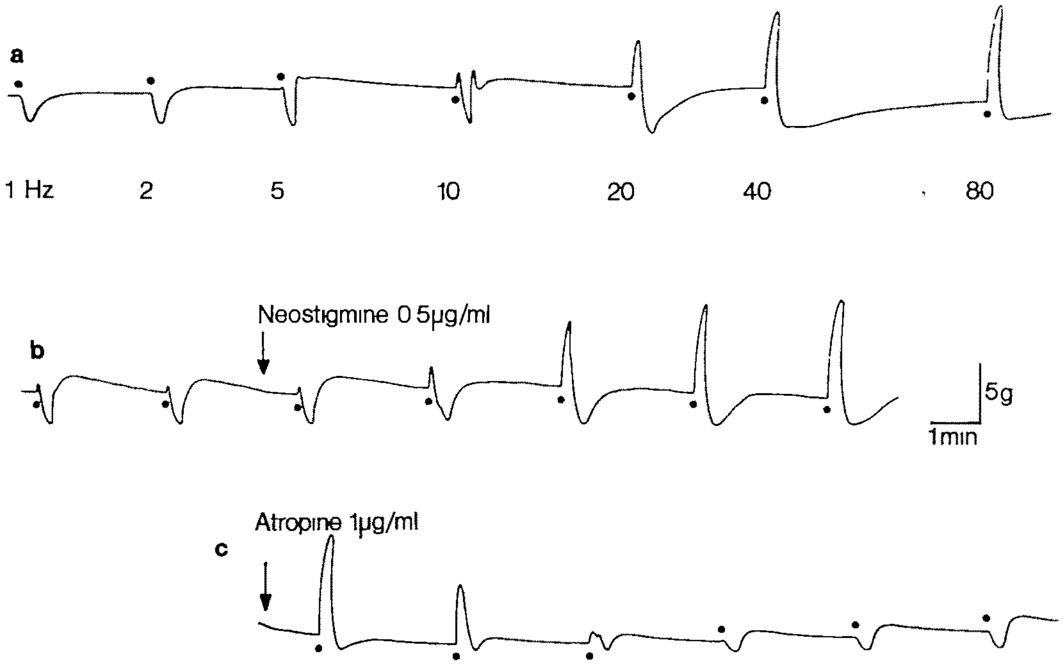

**Figure 1** Relationships between frequency of transmural stimulation (TMS) and (a) types of responses and (b) effects of neostigmine and (c) atropine on contractile response to TMS in the longitudinal smooth muscle from the pig stomach. The records obtained from the same preparation are shown. TMS was applied at various frequencies (a) or 20 Hz (b,c) for 10 s every 3 minutes. Numbers under trace (a) refer to frequency of TMS and (●) indicate points of the stimulation. The horizontal bar shows the scale for 1 min and the vertical bar the scale for 5 grams.

$n=18$). However, when the frequency was increased over 10 Hz, the response became somewhat variable. The response was classified into the following four types: initial rapid contraction followed by relaxation with after-contraction ($n=3$); the same without after-contraction ($n=5$); relaxation followed by after-contraction ($n=7$); pure relaxation ($n=3$). The initial contractile component observed during stimulation also reached a maximum amplitude at 40–80 Hz (Figure 1a).

The relationships between stimulation frequency (1–40 Hz) and relaxation response were also examined after treatment with atropine (0.5–1.0 μg/ml). In ten preparations, TMS elicited a relaxation over the range of frequency from 1–40 Hz. In general, the inhibitory responses were more marked, the greater the tone. Figure 2 shows the depth of relaxations increased with an increase in frequency until it reached a maximum at about 5–10 Hz. Sometimes, maximum relaxation occurred at 2 Hz. When the frequency was increased more than 5 Hz, the depth of the relaxation usually remained fairly constant, and the response was followed by a long-lasting after-contraction in eight preparations (Figure 2a). In two other cases, the relaxations were observed over the frequencies used (Figure 2b). The after-contraction began to develop at the end of each stimulus and increased in amplitude with increasing frequencies of up to 20–40 Hz.

All the responses described so far may be mediated by stimulation of the intramural nerve because TMS with a short pulse duration was effective. Thus, further experiments were carried out to study the nature of the neurones and the transmitter substances which might be involved in producing the present response.

### *Excitatory pathway*

ACh (0.1–10 μg/ml) or carbachol (10 ng–1 μg/ml) contracted the preparations, depending on the concentrations used. TTX (0.25 μg/ml) abolished the contractile response caused by TMS in low tone preparations, but did not affect that induced by ACh. Hexamethonium (100 μg/ml) had no significant effect on the contraction induced by TMS. The contractions in low tone preparations and the initial brief excitatory component of biphasic or triphasic response in tonic preparations caused by TMS, and the contractions produced by ACh were greatly enhanced by neostigmine (0.1–0.5 μg/ml, $n=7$, Figure 1b) and

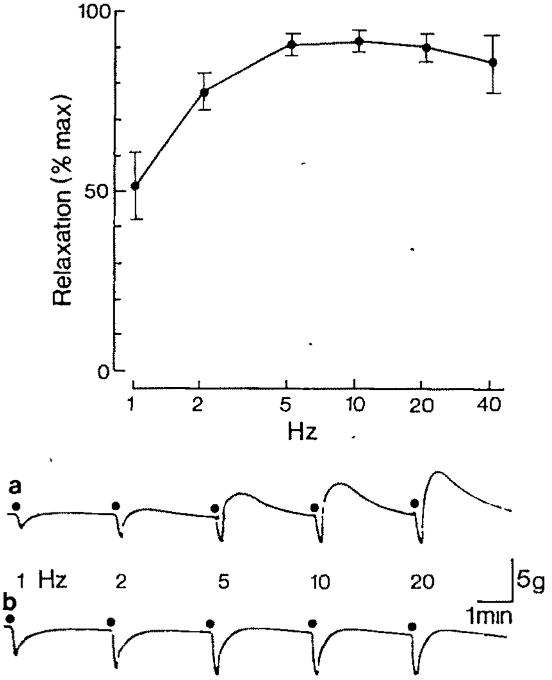

**Figure 2** Relationships between frequency of transmural stimulation (TMS) and amplitude of relaxation of the longitudinal smooth muscle from the pig stomach (in the presence of atropine 0.5 μg/ml). Ordinate scale: amplitude of relaxations expressed as a percentage of the maximum relaxation. Each point is the mean of 10 preparations. Vertical bars show s.e. means. Abscissa scale: frequency of TMS on a logarithmic scale. Traces (a,b): two types of responses elicited by TMS. TMS was applied at various frequencies for 10 s every 3 min; (●) indicate points of the stimulation. The horizontal bar shows the scale for 1 min and the vertical bar the scale for 5 grams

abolished by atropine (0.5–1.0 μg/ml, $n=8$, Figure 1c). After application of atropine, pure relaxations or relaxations followed by after-contractions were observed in response to TMS (Figures 1c and 2a,b).

### *Inhibitory pathway*

In order to analyze the inhibitory responses, the following experiments were performed in the presence of atropine (0.5–1.0 μg/ml). Both TTX (0.25–0.5 μg/ml, $n=8$) and cocaine (50–100 μg/ml, $n=10$) reversibly abolished the inhibitory responses induced by TMS but not those induced by adrenaline (0.25–1.0 μg/ml, Figure 3) and isoproterenol (1 μg/ml).

Nicotine (5–50 μg/ml, $n=23$) and DMPP (5–20 μg/ml, $n=21$) relaxed the preparations in a dose-dependent manner, and the relaxations were also sensitive to TTX and cocaine. Generally, the relaxations developed after a delay of about 10 s and reached their maximum response within a minute.

As shown in Figure 3, the inhibitory responses produced by TMS were little affected by hexamethonium (100–250 μg/ml, $n=11$), which completely abolished the relaxations induced by the ganglionic stimulants ($n=5$). Furthermore, the relaxation in response to TMS and nicotine or DMPP was entirely resistant to $\alpha$-adrenoceptor blocking agents (phenoxybenzamine 0.5–2.0 μg/ml, $n=3$, dibenamine 5.9 μg/ml, $n=2$) and $\beta$-blockers (propranolol 1–5 μg/ml, $n=19$, OPC-1085 1–2 μg/ml, $n=3$) or the combined use ($n=18$) of $\alpha$- and $\beta$-blockers and an adrenergic neurone blocking agent (guanethidine 1–5 μg/ml, $n=10$) (Figures 3 and 4). However, the combined use of $\alpha$- and $\beta$-blocking agents reduced or abolished the relaxations caused by adrenaline (Figure 4) or other sympathomimetic drugs.

The relaxations in response to TMS (0.5–5 Hz) and to ganglionic stimulants were not potentied by cocaine (2–25 μg/ml, $n=7$). Tyramine (2–400 μg/ml, $n=10$) was ineffective in producing the relaxations.

Figure 5a shows that a single pulse of 0.3 ms width or less with a supramaximal intensity gave a transient hyperpolarization of up to 5 mV in the quiescent preparations in the presence or absence of atropine (1 μg/ml, $n=8$). The latency varied between 150–300 ms, and the time required to reach a maximum hyperpolarization was approximately 500 ms or more. When repetitive stimulation (1–10 Hz) was applied to the preparations, individual hyperpolarizations summed with each other to give a larger hyperpolarization (Figure 5a). The amplitude and the rate of hyperpolarization of up to about 15 mV was produced at 10 Hz, the highest frequency used. The hyperpolarization was associated with a relaxation, the amplitude of which was proportional to the size of the hyperpolarization. When repetitive stimulation with a frequency of over 5 Hz was terminated, the membrane potential was reversed transiently to a depolarization of a few mV. At this period, the recovery of the relaxation was facilitated, and after-contractions were often observed.

As shown in Figure 5b, the hyperpolarization was reversibly abolished by TTX (0.25–0.5 μg/ml, $n=4$) and cocaine (50–100 μg/ml, $n=2$), but not significantly affected by hexamethonium (100 μg/ml, $n=2$), guanethidine (1 μg/ml, $n=4$) and the combined use ($n=4$) of phenoxybenzamine (0.5 μg/ml) and OPC-1085 (1 μg/ml).

These pharmacological and electrophysiological analyses suggested that the smooth muscle was supplied with non-adrenergic inhibitory nerves, and that hyperpolarization evoked by TMS is an inhibitory junction potential (i.j.p.) resulting from the excitation of the inhibitory nerves.

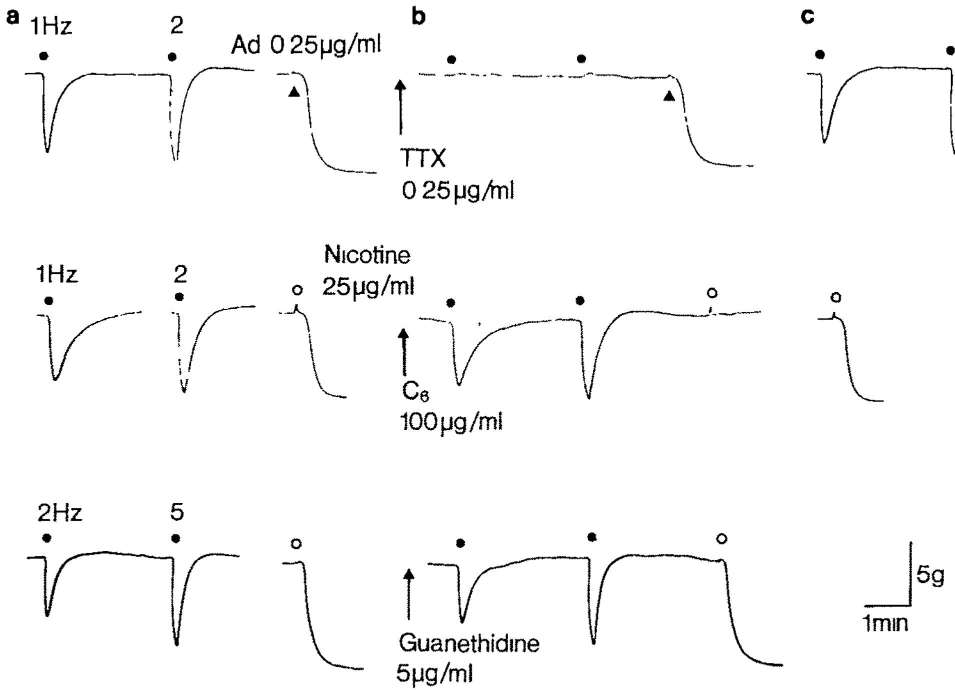

**Figure 3** Effects of tetrodotoxin (TTX), hexamethonium ($C_6$) and guanethidine on inhibitory responses to transmural stimulation (TMS), nicotine and adrenaline (Ad) of the longitudinal smooth muscle from the pig stomach (in the presence of atropine 0.5 µg/ml). (a) Control responses to TMS (●), nicotine (25 µg/ml, ○) and Ad (0.25 µg/ml, ▲). (b) Responses after treatment with TTX (0.25 µg/ml), $C_6$ (100 µg/ml) and guanethidine (5 µg/ml). (c) Responses after washing out TTX and $C_6$ TMS was applied for 10 s every 3 min, and the preparations were exposed to each agonist for 60–90 seconds The horizontal bar shows the scale for 1 min and the vertical bar the scale for 5 grams.

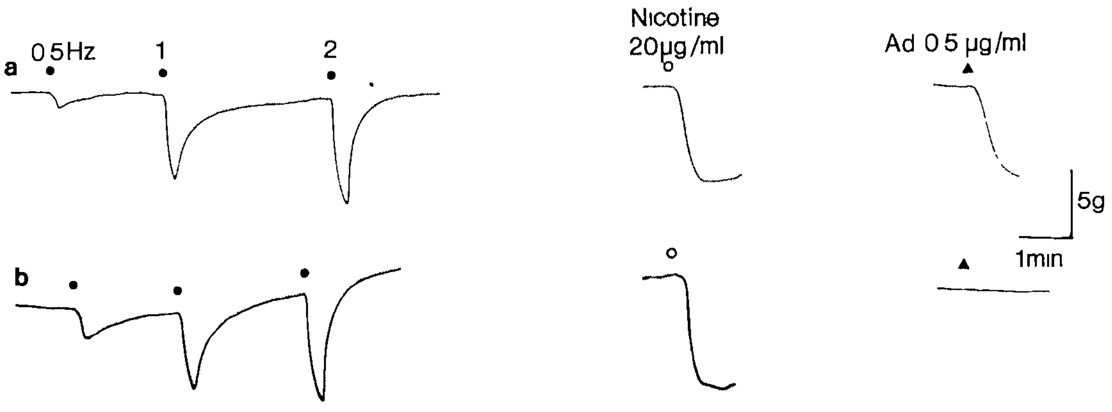

**Figure 4** Effects of phenoxybenzamine plus OPC-1085 on inhibitory responses to transmural stimulation (TMS), nicotine and adrenaline of the longitudinal smooth muscle from the pig stomach (in the presence of atropine 0.5 µg/ml). (a) Control responses to TMS (●), nicotine (20 µg/ml, ○) and adrenaline (Ad, 0.5 µg/ml, ▲) (b) Responses after treatment with phenoxybenzamine (0.5 µg/ml) and OPC-1085 (2 µg/ml). TMS was applied for 10 s every 3 min and the tissues were exposed to the agonist for 90 seconds The horizontal bar shows the scale for 1 min and the vertical bar the scale for 5 grams.

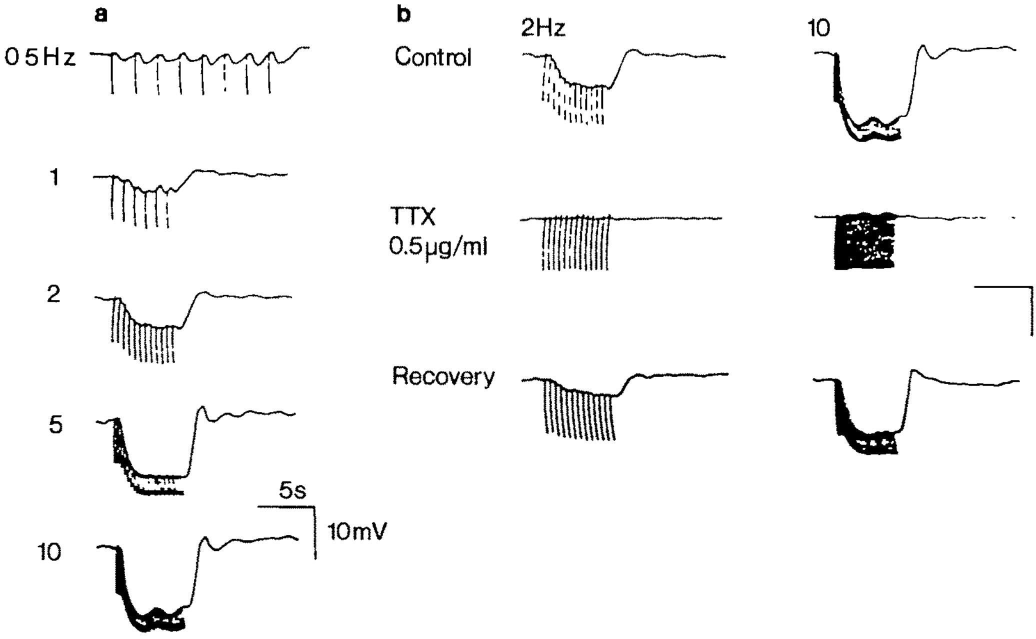

**Figure 5** Sucrose-gap records from the longitudinal smooth muscle of the pig stomach. The records from the same preparation are shown. (a) Hyperpolarization induced by transmural stimulation (TMS) at different frequencies (0.5–10 Hz). (b) Effect of tetrodotoxin (TTX, 0.5 μg/ml) on the hyperpolarization. TMS with a brief pulse duration (<0.3 ms) was applied at the various frequencies (0 5–10 Hz) for about 5 seconds. The horizontal bar shows the scale for 5 s and the vertical bar the scale for 10 mV.

*Comparison between effects of transmural stimulation and exogenously applied ATP or its related compounds*

Burnstock and his colleagues have proposed that the neurohumoral transmitter of the intramural non-adrenergic inhibitory neurones in the gut is an adenine nucleotide, probably ATP. If this is the case, exogenously applied ATP should mimic the response caused by the stimulation of the inhibitory neurones in this prearation. To make certain of this, the responses to TMS and to ATP or its related compounds were compared in the presence of atropine (0.5–1.0 μg/ml) plus guanethidine (1 μg/ml).

There was a distinct difference between the responses to adenine nucleotides or to adenosine and those to the inhibitory nerve stimulation. As illustrated in Figure 6a, ATP (50 μM) caused only a contractile response, despite the fact that TMS at a lower frequency (<5 Hz) produced a marked relaxation.

ATP at a concentration lower than 2 μM elicited no response at all in any of the 15 preparations. ATP (5–20 μM) evoked only a contractile response in 46 out of 70 observations. A threshold dose to induce a contraction was usually between 5 to 10 μM. In general, the contractile response consisted of a tonic contraction with or without spontaneous activity superimposed on it. The contractions began slowly, developed gradually, and attained a peak amplitude within 1–2 minutes. In 24 other cases, ATP in these concentrations induced no response. With a higher dose of ATP (25–200 μM), in addition to a pure tonic contraction, a biphasic response (a tonic contraction preceded by a slight relaxation) was observed. The tonic contractile response to ATP in this range of concentrations was observed in over 57% of the responses, and the biphasic response was noted in 35% of the 153 observations. Such biphasic responses were observed predominantly with a much higher dose of ATP (500 μM or more, in 17 out of 18 observations). The dose-dependency of the slight relaxation in the biphasic response, however, was not clear. TTX (0.5 μg/ml, $n=4$) did not affect the contraction or the biphasic response to ATP. Thus, the responses appeared to be mediated by the direct action of ATP on the smooth muscle.

In contrast to the effects of ATP, TMS at lower frequencies (0.5, 1 and 2 Hz) caused a pure relaxation in about 70% and biphasic responses (marked relaxations followed by after-contractions) in about 30% of the 103 observations. In response to TMS at higher frequencies (5, 10, 20 and 40 Hz) relaxation was observed in 39% of the cases, and a biphasic response in 61% of the 117 observations.

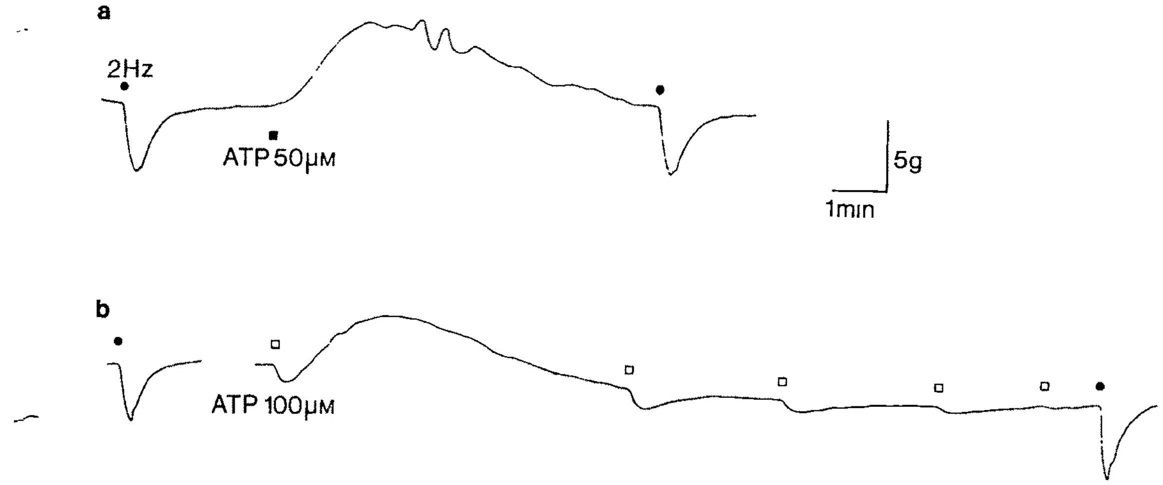

**Figure 6** Dissimilarity between effects of transmural stimulation (TMS) and ATP in the presence of atropine (1 μg/ml) and guanethidine (2 μg/ml). (a) ATP (50 μM, ■) caused a tonic contraction while TMS (●) with a frequency of 2 Hz evoked only a relaxation (b) Effects of desensitization to ATP (100 μM, □) on relaxation induced by TMS. The horizontal bar shows the scale for 1 min and the vertical bar the scale for 5 grams. Note that the inhibitory response induced by TMS was observed without any modification, even after the biphasic responses caused by ATP were completely abolished.

The effects of ADP, AMP and adenosine were also observed under similar experimental conditions. ADP (25–200 μM) caused a tonic contractile response or a biphasic one. The percentage appearance of the contractile response and the biphasic response to ADP ($n=53$) was about the same as that caused by ATP. The same AMP and adenosine doses elicited only a contractile response in all 40 and 49 observations, respectively. The contractile responses to ADP, AMP and adenosine were also dose-dependent, and there was no significant qualitative difference in their time course. The order of potency of adenine nucleotides and adenosine in their ability to elicit contractile responses was ADP > ATP > AMP > adenosine (Figure 7).

Figure 8 shows the change in membrane potential due to the application of ATP. In the spontaneously active preparations, ATP (50–100 μM) increased the frequency of spike discharges accompanied by tension development (Figure 8a). In the quiescent muscles, ATP caused depolarization of the cell membrane,

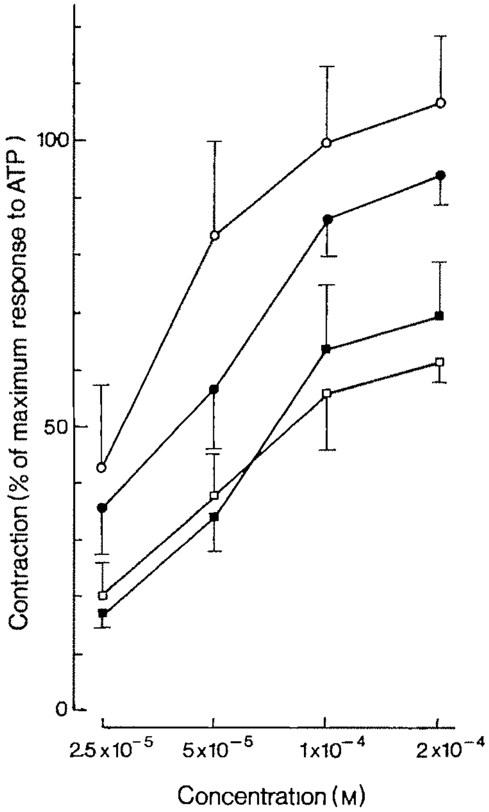

**Figure 7** Dose-response curves for adenine nucleotides and adenosine in causing contractions of longitudinal smooth muscle from the pig stomach. Ordinate scale: amplitude of contractions expressed as a percentage of the maximum contraction to ATP. Each point is the mean of at least 6 preparations. Vertical bars show s.e. mean. Abscissa scale: molar dose of the drugs. Dose-response curves are shown for ATP (●), ADP (○), AMP (■) and adenosine (□).

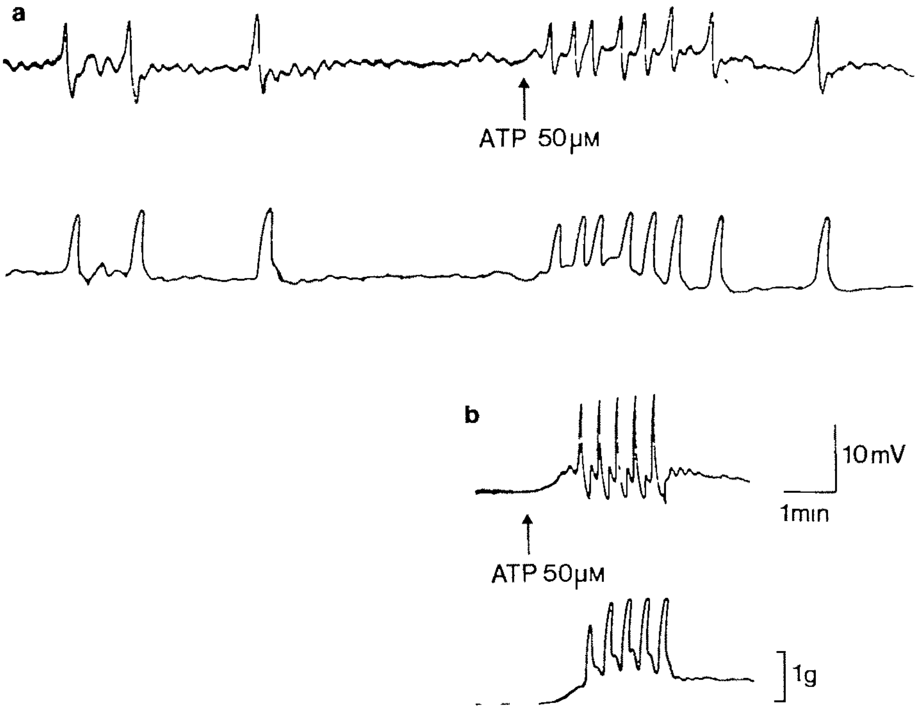

**Figure 8** Sucrose-gap records from the longitudinal smooth muscle of the pig stomach showing excitatory responses to ATP (50 μM) in (a) spontaneously active preparations and in (b) quiescent preparations. Upper trace: membrane potential. Lower trace: tension. Arrows refer to points of the drug application. The vertical bars are the scale for 10 mV and 1 g, and the horizontal bar the scale for 1 min, respectively.

which was usually superimposed with action potentials (Figure 8b). The depolarization was followed by a tonic contraction. When action potentials were generated, phasic contractions were superimposed upon the tonic contraction.

### *Tachyphylaxis to ATP*

It has been reported that stimulation of non-adrenergic inhibitory nerves fails to induce the inhibitory responses of the rabbit ileum which has been desensitized to ATP by exposure to a high dose of the drug (Burnstock *et al.*, 1970). This result is considered as evidence supporting the purinergic nerves hypothesis. In the rabbit ileum, however, exposure to high concentrations of either ATP or adenosine desensitized the preparation to ATP, ADP, AMP and adenosine, but the inhibitory responses to the intramural or sympathetic nerve stimulation and to sympathomimetic agents were not affected (Weston, 1973). We, therefore, investigated whether the pig stomach preparations could be desensitized to ATP and TMS at the same time.

Desensitization to ATP could be quite easily achieved in this preparation by repeated administration of ATP (50–100 μM, $n=6$) at short intervals without washing out the bath. As shown in Figure 6b, a long-lasting tonic contraction preceded by a slight relaxation disappeared more quickly than the inhibitory response. Even after a second application of ATP at intervals of 2–3 min, a further addition of ATP failed to cause the contractile response. The residual inhibitory component also decreased in amplitude and disappeared after a few subsequent administrations of the drug. When the ATP desensitization of the preparations was completed, however, the TMS (1–5 Hz) still evoked a relaxation without any significant modification in the presence of atropine plus guanethidine (Figure 6b). The effect of desensitization to ATP was abolished by washing out the bath solution for about 30 minutes.

### *Effect of dipyridamole*

Satchell *et al.* (1972) have reported that dipyridamole and hexobendine selectively potentiated the inhibitory response of the guinea-pig taenia coli to non-adrenergic nerve stimulation simultaneously with ATP. We also attempted to observe the effect of dipyridamole on the responses to TMS and ATP and

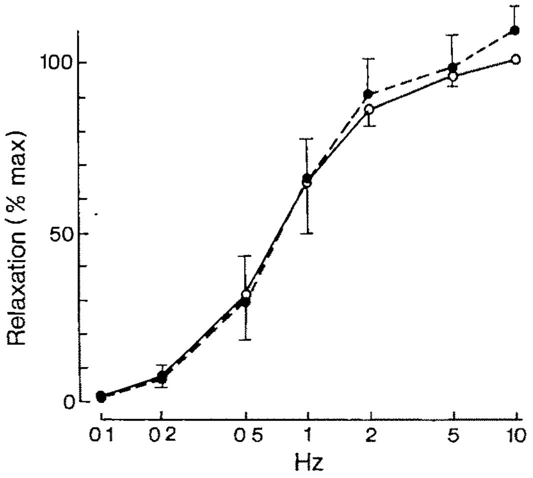

**Figure 9** Effects of dipyridamole on inhibitory responses caused by transmural stimulation (TMS) at various frequencies in the presence of atropine (0.5 µg/ml) and guanethidine (1 µg/ml). Ordinate scale: amplitude of relaxations expressed as a percentage to the maximum relaxation Each point is the mean of 5 preparations. Vertical bars show s e mean Abscissa scale: frequency of TMS on a logarithmic scale Frequency-response curves are presented for the control (O), and for that after treatment with dipyridamole (0.2–1 µg/ml, ●).

noradrenaline. As illustrated in Figure 9, the inhibitory response to TMS (0.1–10 Hz) in the presence of atropine and guanethidine was not potentiated by dipyridamole (0.2–1 µg/ml) in any of the 15 cases. Furthermore, dipyridamole did not cause any consistent effect on the responses to ATP (20–200 µM, $n=18$). More specifically, the tonic contractions ($n=13$) or the contractile components of the biphasic responses ($n=5$) caused by ATP were unaffected ($n=9$), potentiated ($n=4$) or inhibited ($n=5$), and the inhibitory components remained unaffected in all of the observations. The effect of dipyridamole on the inhibitory response to noradrenaline (0.5 µg/ml, $n=6$) was also variable, i.e., it was reduced or reversed to a contractile response.

## Discussion

The present experiments show that the longitudinal smooth muscle of the pig stomach is innervated at least with intrinsic cholinergic excitatory and non-adrenergic inhibitory nerves, and ATP (or its related compounds) is probably not the transmitter substance in the inhibitory neurones.

Biphasic responses induced by TMS were reversibly abolished by TTX and cocaine, but they were not significantly affected by hexamethonium. The contractile component of the response was enhanced by neostigmine and abolished by atropine. After treatment with atropine, TMS and ganglionic stimulants consistently produced a relaxation. The physiological and pharmacological properties of the relaxation were similar to those caused by excitation of the non-adrenergic inhibitory neurones in the atropinized gut smooth muscle preparations isolated from various animals (see list of references in Burnstock, 1972). The similarity was that stimulation at a low range of frequencies and ganglionic stimulants were effective in causing a relaxation; either a single or repetitive stimulation (<10 Hz) caused hyperpolarization (i.j.p.). All the mechanical and electrical responses were insensitive to the adrenergic neurone blocking agent, and to $\alpha$- and $\beta$-adrenoceptor blocking agents. From these similarities, it may be suggested that the nerves responsible for inducing the relaxations of the present preparation are similar to the intramural non-adrenergic inhibitory neurones, as reported for various other preparations.

There was no parallelism between the effects of stimulation of non-adrenergic inhibitory neurones and of exogenously applied ATP or its related compounds in the present preparation. In the presence of atropine and guanethidine, ATP (5–20 µM), AMP and adenosine (25–200 µM) caused only a tonic contraction, despite the fact that TMS with low frequencies (>5 Hz) usually produced a marked relaxation. The contractile response induced by ATP was always preceded by membrane depolarization. However, both ATP and ADP in a higher concentration of 25–200 µM, caused a tonic contraction preceded by a slight relaxation in 35% out of the 153 and 53 observations, respectively. The incidence of a marked relaxation with an after-contraction was increased by higher frequency stimulation (<5 Hz) of the intramural inhibitory nerves. Burnstock *et al.* (1970) have pointed out that there may exist two different ATP receptors for excitation and for inhibition in the gastrointestinal smooth muscle. The contractions elicited by adenine nucleotides and adenosine in this preparation increased in amplitude in a dose-dependent manner, but the relaxation did not. On the other hand, the amplitude of relaxation induced by TMS was increased with an increase in frequency until it reached a maximum at about 5–10 Hz. In addition, even with a high concentration such as 1 mM, ATP never produced a relaxation having a comparable amplitude with those caused by TMS at the optimum frequency (1–5 Hz). The dissimilarity between the response induced by stimulation of the intramural inhibitory nerve and that induced by ATP or its related compounds has been reported in the longitudinal smooth muscle of the rabbit rectum (MacKay & McKirdy, 1972) and in the rat stomach (Heazell, 1969, cited by Furness & Costa, 1973; Burnstock *et al.*, 1970) and in the chicken colon and rectum (Takewaki, Ohashi & Okada, unpublished results). Moreover Rikimaru, Fukushi & Suzuki (1971), Suzuki, Fukushi & Rikimaru (1971) and Saito (1972) have reported that phentolamine and imidazole greatly reduced the relaxations of the guinea-pig taenia coli induced by ATP without affecting those due to the excitation of the non-adrenergic inhibitory nerves.

Furthermore, even after both the inhibitory and excitatory components of the biphasic response to ATP were abolished, TMS was still effective in producing the relaxation without any modification. Similar results have been reported in the rabbit duodenum (Weston, 1973) and chicken colon and rectum (Takewaki, Ohashi & Okada, unpublished results). Quite recently, Hooper, Spedding, Sweetman & Weetman (1974) have reported that 2-2′-pyridylisatogen tosylate (PIT) is a specific antagonist to ATP. This compound was found to inhibit the relaxation of guinea-pig taenia coli elicited by ATP without affecting the inhibitory responses to stimulation of non-adrenergic inhibitory nerves (Spedding, Sweetman & Weetman, 1975).

Satchell *et al.* (1972) and Satchell & Burnstock (1975) have suggested that dipyridamole and hexobendine potentiated the inhibitory responses of the isolated guinea-pig taenia coli induced by both the stimulation of non-adrenergic inhibitory nerves and ATP, by inhibiting the uptake of adenosine. In the present preparation, however, dipyridamole was not found to be effective in either potentiating the inhibitory responses induced by TMS or in causing any consistent effect on the ATP-induced responses. The ineffectiveness of dipyridamole in enhancing the relaxations resulting from the excitation of non-adrenergic inhibitory nerves in the rabbit duodenum (Hulme & Weston, 1974) and in the rat stomach (Heazell, 1975) has also been reported.

It appears, therefore, that the purinergic nerves hypothesis proposed by Burnstock *et al.* (1970) and Burnstock (1972) is not applicable to intramural inhibitory transmission from non-adrenergic inhibitory neurones to the smooth muscle.

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# AN ELECTROPHYSIOLOGICAL STUDY OF THE ACTION OF CRUDE *Enhydrina schistosa* VENOM

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**1** Electrophysiological studies on the rat diaphragm revealed that crude *Enhydrina schistosa* venom has a blocking action on postjunctional acetylcholine (ACh) receptors.

**2** This venom in concentrations of 0.125–1.0 μg/ml abolished the extracellular endplate potential (e.e.p.p.) without altering the presynaptic spike. When the phrenic nerve was stimulated at a frequency of 1 Hz the degree of depression of successive e.e.p.ps was approximately the same in records made before and after treatment with venom.

**3** In concentrations of 0.25 and 0.5 μg/ml the venom reduced significantly the amplitude of miniature endplate potentials (m.e.p.ps). The frequency of m.e.p.ps did not increase but was decreased when the amplitude of the m.e.p.ps was much reduced.

**4** The crude venom did not alter the resting membrane potential (RMP) of the muscle cell.

**5** The venom inhibited the depolarization of the muscle membrane caused by carbachol.

**6** It is concluded that an active component in the crude venom is responsible for a blocking action on postjunctional receptors.

## Introduction

The crude venom of *Enhydrina schistosa*, a sea snake found in Malaysian waters, has been shown to paralyse skeletal muscle by blocking transmission across the neuromuscular junction (Carey & Wright, 1961; Cheymol, Barme, Bourillet & Roch-Arveiller, 1967; Geh, 1968). Attempts made to elucidate its site of action at the neuromuscular junction have revealed a presynaptic effect with relatively small doses (Geh & Chan, 1973; Toh, Geh & Chan, 1975); however, an action on postjunctional receptors by the crude venom has been suggested (Carey & Wright, 1961; Cheymol *et al.*, 1967; Geh, 1968). Since it is possible for crude snake venoms to have two main sites of action (Chang & Lee, 1963; Datyner & Gage, 1973a,b) the present electrophysiological study was designed to determine whether the action of crude *E. schistosa* venom is pre- or postjunctional or even both.

## Methods

The phrenic nerves and diaphragms were dissected from Wistar rats of either sex weighing 140–200 grams. The rats were killed by a blow on the head followed by section of the spinal cord in the neck. Both right and left hemidiaphragms were used and were bathed in a modified Liley's (1956a) solution of the following composition (mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ (aq) 1, $NaH_2PO_4$ (aq) 1, $NaHCO_3$ 12 and dextrose 25, aerated with 5% $CO_2$ in $O_2$.

The arrangements for extracellular and intracellular recording from the preparation have been described by Ferry & Marshall (1973). The diaphragm was pinned down in a perspex bath and illuminated from below. Medium flowed into the bath at approximately 1 ml/min and the amount in the bath was kept at 20 ml by a constant level device and at 37 ± 1 °C. The phrenic nerve was stimulated through a pair of silver electrodes mounted in a perspex tube with pulses 5x threshold strength, with a pulse width of 0.05 ms and at 0.1 Hz. The bathing medium contained 1.3 μM (+)-tubocurarine chloride. Extracellular recordings (e.e.p.p.) were made with insulated silver wire electrodes (0.13 mm diam., diamel coated, Johnson,

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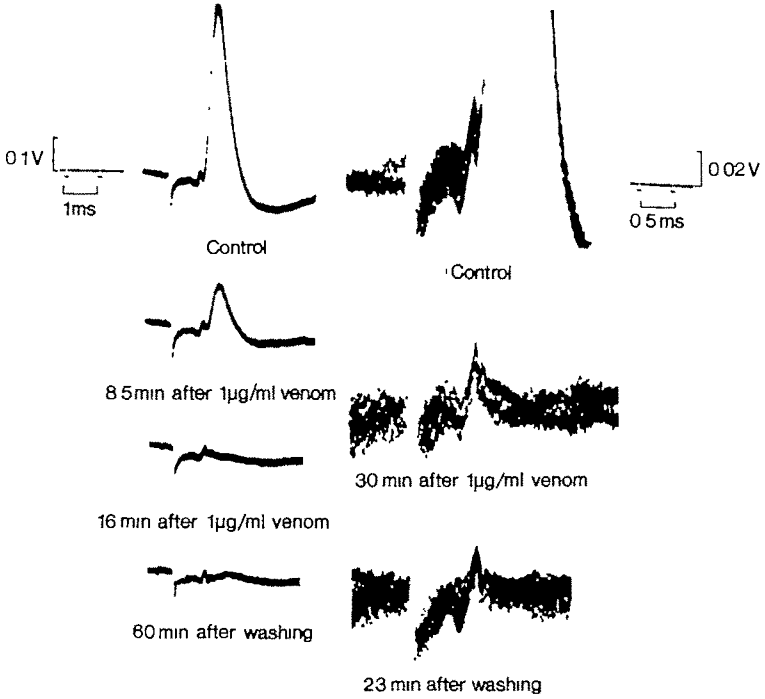

**Figure 1** Extracellular records of activity at endplate region of rat phrenic nerve-diaphragm preparation 37°C (1.3 μM (+)-tubocurarine), showing (left) presynaptic spike followed by endplate potential, and (right) the similar records at 5x gain to show presynaptic spike, each trace being 10 superimposed responses

Matthey & Co.) suitably supported in glass capillary tubings. One electrode was located at the endplate region, the indifferent electrode being placed in the medium. The endplate region was located in blocked preparations by positioning the electrode so that the amplitude of the endplate potential and its rate of rise were maximal and a presynaptic spike was seen. The preamplifier had a bandwidth of 0.2 Hz–10 kHz.

For intracellular recording of miniature endplate potentials (m.e.p.ps) and resting membrane potentials (RMP) the glass microelectrodes were filled with 3M KCl and had a tip resistance of 10–30 MΩ. The recording system had a bandwidth of 0.8 Hz–1 kHz for m.e.p.ps and was directly coupled for recording RMP.

### *Drugs*

Crude *Enhydrina schistosa* venom (dried in a dessicator) was obtained from the Institute of Snake and Venom Research, Penang; (+)-tubocurarine chloride from Burroughs Wellcome and carbamylcholine chloride from BDH. All stock solutions of the drugs used were prepared in double distilled water, but final dilutions were in Liley's solution.

## Results

### *Effect of* E. schistosa *venom on extracellular endplate potentials*

In 10 experiments endplate potentials were recorded extracellularly in preparations which had been treated with (+)-tubocurarine 1.3 μM for at least 1 h to achieve a steady block of neuromuscular transmission.

After the addition of *E. schistosa* venom within the range 0.125–1 μg/ml, the amplitude of the extracellular endplate potential decreased. The extent of the decrease and its time course depended on the dose of venom, and was maximal within 15–40 minutes. The presynaptic spike remained despite considerable changes in the e.e.p.p. (Figure 1). Washing the preparation with fresh Liley's solution and/or stopping the stimulation did not restore the e.e.p.p. nor have any marked effect on the presynaptic spike. The persistence of the presynaptic spike affords an indication that the reduction of the e.e.p.p. is not artefactual; for instance, it is not due to the electrode moving off the original focus of activity.

It is concluded that *E. schistosa* venom does not affect the conduction of the presynaptic action

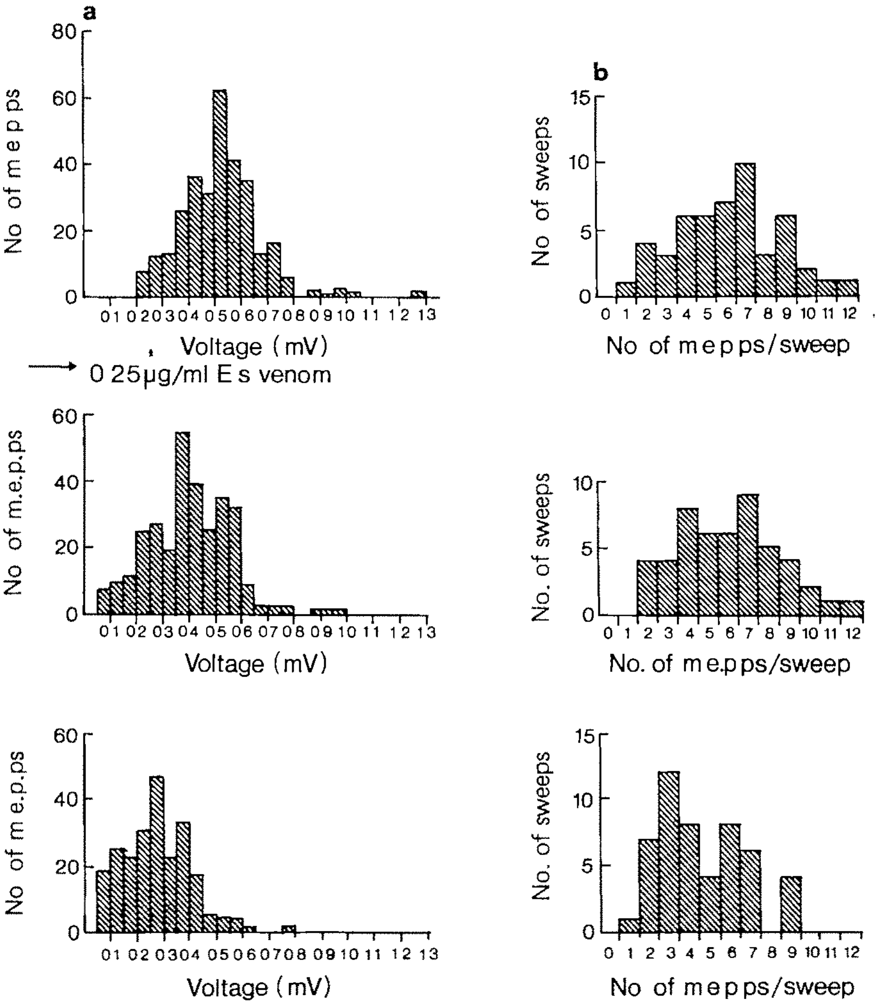

**Figure 2** Miniature endplate potentials of rat phrenic nerve-diaphragm preparation (37°C) recorded intracellularly (a), Histograms of m.e.p.p. amplitude; (b) histograms of number of m.e p p s per 0.5 s sweep. All histograms constructed from 50 sweeps at 1 Hz. The preamplifier was set at a bandwidth of 0.8 Hz–1 KHz. Top: control conditions; Middle: 4 min after *Enhydrina schistosa* (E.s.) toxin 0 25 µg/ml, Lower: 9 min after E.s. toxin 0 25 µg/ml.

potential, but interferes with the release or action of acetylcholine.

### *Effect of* E. schistosa *venom on tetanic rundown of extracellular endplate potentials*

In 10 preparations experiments were made on the effect of *E. schistosa* venom on the run-down at 1 Hz of the e.e.p.p. Before adding the venom, the 5th e.e.p.p. was 83% of the first (s.e. ± 1.47). After venom in concentrations of 0.125–1 µg/ml there was no significant change in the rate of tetanic run-down, although the amplitudes of the e.e.p.ps were reduced, as described above.

These experiments suggested there was no change in the fractional release of transmitter from the nerve terminals after *Enhydrina schistosa* venom.

### *Effect of* E. schistosa *venom on the resting membrane potential*

In each of 10 preparations the membrane potential of 30 cells was measured, the preparation was exposed to 2.5 µg/ml of venom and 30 min later the membrane potentials of a further 30 cells was measured. At this time there was no response of the muscle to stimulation of the nerve. The overall mean and s.e. was calculated from the mean values in individual preparations. This overall mean and s.e. for control values was 70.61 mV (s.e. ± 1.45) and after the venom the overall mean was 71.60 mV (s.e. ± 1.05). There is no significant difference between these values ($P > 0.05$). It is concluded that *E. schistosa* venom has no significant effect on the membrane potential.

### *Effect of* E. schistosa *venom on miniature endplate potentials*

Miniature endplate potentials were displayed on an oscilloscope with sweep time of 0.5 s and photographs were taken of successive sweeps. M.e.p.ps were recorded before and after the application of venom at 0.25 µg/ml or 0.5 µg/ml. The distribution of amplitude of m.e.p.ps was prepared from the photographs (Figure 2, left). The distribution of the number of m.e.p.ps in each sweep was also prepared as an index of m.e.p.p. frequency (Figure 2, right). The amplitude of m.e.p.ps began to decrease within 3–5 min, and in Figure 2 where sampling began 4 min after the administration of venom, there was a significant reduction of m.e.p.p. amplitude compared to control ($P \leqslant 0.001$) without any marked change in their frequency distribution. When sampling began 9 min after the application of venom, there was a further reduction in m.e.p.p. amplitude with an apparent reduction in m.e.p.p. frequency. It is considered that the apparent reduction of m.e.p.p. frequency is a reflection of the reduction of some m.e.p.p. amplitudes to within the noise of the recording system. It is concluded that *E. schistosa* venom reduces m.e.p.p. amplitude and is likely to have little or no effect on the frequency of release of quanta of transmitter.

### *Effect of* E. schistosa *venom on depolarizing effect of carbachol*

Experiments were done on 11 preparations in which the effect of venom on the carbachol-induced depolarization of muscle cells was investigated. In control preparations, membrane potentials of about 25–30 cells per diaphragm were sampled. The preparations were exposed to carbachol for 30 min and the membrane potentials were sampled again. The effects of 1 µM to 1 mM concentrations of carbachol were measured.

In the treated preparations the membrane potentials of 30 cells per diaphragm were sampled before and after 2.5 µg/ml venom, there being no significant change, as mentioned previously. *E. schistosa* toxin at 2.5 µg/ml was used to give a rapid large and irreversible effect (Geh, 1968). The toxin-treated preparations were then exposed to carbachol for 30 min before sampling of the membrane potentials was carried out.

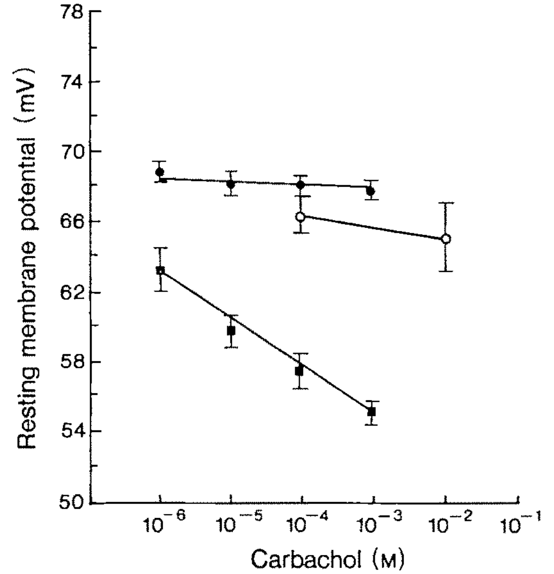

**Figure 3** Membrane potential at the endplate region of rat diaphragm (37°C). (●) Control values of membrane potential measured throughout the experiment and related to membrane potential in presence of carbachol (■) Membrane potential recorded for each concentration of carbachol in bathing fluid (○) Membrane potential recorded for each concentration of carbachol added to bathing fluid already containing *Enhydrina schistosa* venom 2.5 µg/ml. Each point represents mean of membrane potentials sampled from 150–180 cells from a total of 5–6 diaphragms. Vertical lines show s e mean.

The results of these experiments are summarized in Figure 3. The application of *E. schistosa* toxin resulted in a smaller effect of carbachol, the dose-ratio being greater than $10^4$. It is concluded that *E. schistosa* toxin reduces the effect of carbachol on the endplate.

## Discussion

Effective blocking doses (Geh, 1968) of crude *Enhydrina schistosa* venom abolished the e.e.p.p. progressively without having much effect on its presynaptic spike potential (Figure 1). Since this spike reflects activity in the motor nerve terminal (Eccles, Katz & Kuffler, 1941; Hubbard & Schmidt, 1963; Katz & Miledi, 1965a), its integrity suggests that the terminal depolarization resulting in activated sodium conductance is probably not affected by the venom. However, the depolarization-secretion coupling that is responsible for the release of the transmitter (Katz & Miledi, 1965b) may or may not have been changed. A block of postjunctional receptors may be responsible for the progressive disappearance of the e.e.p.p. The tetanic rundown experiments do not suggest an effect of *E. schistosa* venom on transmitter release and therefore tend to support the hypothesis of a postjunctional action.

The action of the venom on m.e.p.ps lends more support to its postjunctional action on the receptors. Here the amplitude of the m.e.p.ps progressively declined but their frequency showed little change even when there was already a significant fall in potential. Since frequency change can reflect only presynaptic events (del Castillo & Katz, 1954; Liley, 1956b), the absence of changes indicates that the venom probably does not have presynaptic action on transmitter release. The fall in frequency that occurred late in the experiment may be due to the loss of potentials below the noise level as the potential amplitude fell. The fall in m.e.p.p. amplitude itself may reflect both pre- and postsynaptic events; however, more likely, postsynaptic ones (Hubbard, Llinas & Quastel, 1969). In our experiments rapid decline of potential amplitude with time (Figure 2a) indicates an action of the venom blocking the postjunctional receptors, thereby preventing acetylcholine action, rather than a decrease in the quantal content of acetylcholine (Elmqvist & Quastel, 1965), for if the venom interfered with the synthesis of acetylcholine resulting in depletion of acetylcholine stores, one would expect the change in amplitude to occur only after prolonged stimulation.

The shift to the right of the dose-response curve with carbachol (Figure 3) supports the possibility of a postsynaptic action. The progressive and irreversible nature of *E. schistosa* venom block was demonstrated in our present experiments and has often been shown previously (Carey & Wright, 1961; Cheymol *et al.*, 1967; Geh, 1968; Geh & Chan, 1973).

From electrophysiological studies, it appears therefore that effective paralysing doses of crude *E. schistosa* venom probably exert an effect by combining irreversibly with the postjunctional receptors without altering significantly the resting membrane potential of the muscle cells. Nevertheless, an effect of the crude venom on the motor nerve terminal, whether prior to or together with, its action on the postjunctional membrane cannot be ruled out entirely. Geh & Chan (1973) have suggested that in the cat, relatively small non-paralysing doses of the venom could possibly have an initial prejunctional effect whereas effective blocking doses have been thought to combine irreversibly with postjunctional receptors. Moreover Toh *et al.* (1975) have shown that after chronic treatment in guinea-pig, sublethal doses of crude *E. schistosa* venom caused destruction of mitochondria in the motor nerve terminal. However, no gross evacuation of synaptic vesicles or changes in the axonal membrane were seen. Since all these studies have been carried out with the crude venom it is not surprising that manifestations of various effects have been indicated depending on dose, experimental animals and techniques. Pre- and postjunctional sites of action have been attributed to other crude snake venoms as well (Chang & Lee, 1963; Datyner & Gage, 1973a,b).

It is not possible at this stage to identify which component(s) or combination of active constituents in the crude *E. schistosa* venom that can be responsible for the effects seen now and in previous investigations. The only solution is to study the mode of action of purified active fractions; at present, experiments are being carried out towards this end.

This work was carried out in the Department of Pharmacology, The Medical School, University of Newcastle-upon-Tyne, where S.L.G. was on sabbatical leave. The technical assistance of Mr A. Ward is appreciated.

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# THE ROLE OF $\beta$-ADRENOCEPTORS IN THE RESPONSES OF THE HEPATIC ARTERIAL VASCULAR BED OF THE DOG TO PHENYLEPHRINE, ISOPRENALINE, NORADRENALINE AND ADRENALINE

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**1** The sympathetically-innervated hepatic arterial vascular bed of the dog was perfused from a femoral artery. Hepatic arterial blood flow and perfusion pressure were recorded continuously, and the hepatic arterial vascular resistance (HAVR) calculated from these measurements.

**2** Intra-arterial injections of phenylephrine caused dose-dependent rises in HAVR, indicating hepatic arterial vasoconstriction, at all doses above threshold. No secondary reductions in HAVR followed these responses.

**3** Intra-arterial injections of isoprenaline caused only dose-dependent reductions in HAVR at doses above threshold.

**4** Intra-arterial injections of noradrenaline typically caused an initial increase in HAVR which was followed at all but the highest doses by a secondary, delayed, reduction in HAVR.

**5** Intra-arterial injections of adrenaline, like those of noradrenaline, resulted in hepatic arterial vasoconstriction followed by hepatic arterial vasodilatation.

**6** On a molar basis, the most potent hepatic arterial vasoconstrictor was noradrenaline, followed by adrenaline and phenylephrine.

**7** The maximum reductions in HAVR caused by adrenaline (mean reduction = 21.9%) and noradrenaline (16.9%) were significantly smaller than those due to isoprenaline ($P < 0.001$).

**8** Propranolol attenuated the hepatic arterial vasodilator responses due to isoprenaline, and the secondary falls in HAVR following intra-arterial adrenaline and noradrenaline.

**9** Propranolol did not modify the vasoconstrictor responses to phenylephrine.

**10** Both adrenaline and noradrenaline were more potent hepatic arterial vasoconstrictors after propranolol than in the absence of $\beta$-adrenoceptor blockade. The potentiation of the vasoconstrictor effects of adrenaline was statistically significant.

**11** After propranolol, adrenaline was a more potent hepatic arterial vasoconstrictor than noradrenaline.

**12** Since the $\beta$-adrenoceptors in the hepatic arterial vasculature were not blocked by atenolol, but were stimulated by salbutamol, it is concluded that they are predominantly of the $\beta_2$-type.

**13** The vasoconstrictor actions of phenylephrine, noradrenaline and adrenaline were all antagonized by the systemic administration of phentolamine, all three dose-response curves being shifted to the right.

**14** The results are discussed with regard to the possible control of the hepatic arterial vasculature by naturally-occurring catecholamines.

## Introduction

The responses of the hepatic arterial vascular bed to locally and systemically administered catecholamines have yielded information regarding the adrenoceptor population in the hepatic arterial resistance vessels in various species (Greenway & Stark, 1971). In the dog, the presence of $\alpha$-adrenoceptors mediating hepatic arterial vasoconstriction has been indicated by the responses to locally administered noradrenaline,

adrenaline (Andrews, Hecker, Maegraith & Ritchie, 1955; Green, Hall, Sexton & Deal, 1959; Scholtholt, Lochner, Renn & Shiraishi, 1967; Hanson, 1973; Richardson & Withrington, 1976a and unpublished observations) and phenylephrine (Bender, Larsen & Horvath, 1962). In the cat, the influence of adrenoceptor antagonists on the responses of the hepatic arterial vasculature has also been investigated (Greenway & Lawson, 1969; Ross & Kurrasch, 1969).

The presence of $\beta$-adrenoceptors in the hepatic arterial vasculature has been established in the cat (Greenway & Lawson, 1969) and dog (Richardson & Withrington, 1976b,d) but the possible contribution of $\beta$-adrenoceptor stimulation to the hepatic arterial vascular responses to locally-administered naturally-occurring catecholamines has not been established quantitatively. In order to demonstrate vasodilatation quantitatively in this vascular bed, it is necessary to retain the sympathetic innervation intact (Richardson & Withrington, 1976d; 1977a); if the periarterial nerves accompanying the hepatic artery are sectioned, catecholamines injected intra-arterially cause only vasoconstriction (Richardson & Withrington, 1976a and unpublished observations); similarly, the administration of $\alpha$-adrenoceptor antagonist drugs may reduce the hepatic arterial vasoconstrictor tone which is necessary for the quantitative examination of vasodilator responses.

The present experiments were designed to establish the relative properties of intra-arterially injected phenylephrine, isoprenaline, noradrenaline and adrenaline on the hepatic arterial resistance vessels. The contribution of $\beta$-adrenoceptor stimulation to these effects was analysed by using the nonselective $\beta$-adrenoceptor antagonist, propranolol. These experiments revealed the presence of $\beta$-adrenoceptors which were stimulated to cause hepatic arterial vasodilatation by isoprenaline and also by adrenaline and noradrenaline, but not by phenylephrine. Subsequently, the nature of the $\beta$-adrenoceptors in this vascular bed was examined by using the selective $\beta_2$-adrenoceptor stimulant salbutamol (Daly, Farmer & Levy, 1971) and the selective $\beta_1$-adrenoceptor antagonist atenolol (ICI 66082) (Hainsworth, Karim & Stoker, 1974).

Preliminary reports of parts of this investigation have been presented to the British Pharmacological Society (Richardson & Withrington, 1976b; 1977c).

## Methods

The technique used in these experiments was the same as that reported previously (Richardson & Withrington, 1976d; 1977a) and only brief details of the preparation are given here.

Seventeen dogs weighing between 10.0 and 17.5 kg (13.2 ± 2.1: mean ± s.d.) were deprived of food but allowed access to water *ad libitum* for 24 h before the induction of anaesthesia with methohexitone sodium (Brietal, Lilly); anaesthesia was maintained with chloralose and urethane (50 mg/kg and 500 mg/kg, i.v. respectively). Following a midline laparotomy, the common hepatic artery was dissected free from its accompanying sympathetic nerves which were carefully preserved. The animals were heparinized (250 iu/kg and 100 iu/kg hourly, i.v.) and the hepatic artery cannulated and perfused with blood from a femoral artery. The hepatic arterial mean blood flow and mean perfusion pressure were monitored continuously as described previously (Richardson & Withrington, 1976d). In addition, the systemic arterial blood pressure, heart rate, and inferior vena cava pressure were recorded continuously, to monitor the possible systemic effects resulting from large doses of locally administered drugs which may survive passage through the liver and pulmonary vascular bed to enter the systemic circulation.

### *Presentation of results*

*Hepatic arterial vascular resistance (HAVR)* was calculated as (hepatic arterial mean perfusion pressure; mmHg)/(hepatic arterial mean blood flow; ml/min or ml $\text{min}^{-1}$ 100 $\text{g}^{-1}$). Hepatic arterial vascular resistance was therefore expressed in mmHg $\text{ml}^{-1}$ min, or mmHg $\text{ml}^{-1}$ min 100 g.

*Changes in hepatic arterial vascular resistance* were calculated as percentage changes from the control value immediately before any drug administration to the peaks of the drug-induced effects, i.e. (change in HAVR x 100)/(control HAVR).

Liver weight was obtained immediately *post mortem*; all values expressed per 100 g refer to this terminal weight of liver.

### *Drug administration*

*Agonist agents.* Agonists were injected directly into the hepatic arterial blood stream, in volumes not in excess of 0.5 ml, washed in with 0.9% w/v NaCl solution (saline) to a total injectate volume of 1.5 ml. The drugs used, their molecular weights and the forms in which their weights are subsequently expressed, were as follows: adrenaline bitartrate (Macarthays; mol. wt. 333.3; salt) isoprenaline sulphate (Macarthays; mol. wt. = 556.6; salt), phenylephrine hydrochloride (Boots; mol.wt. = 203.7; salt), noradrenaline acid tartrate (Levophed, Winthrop; mol. wt. of base = 169.0, mol. wt. of salt = 319.3; base), and salbutamol sulphate (Ventolin, Allen & Hanburys; mol. wt. = 288.4; salt).

*Antagonist agents.* Antagonists were administered by intravenous injection in amounts previously found adequate to attenuate the effects of the corresponding

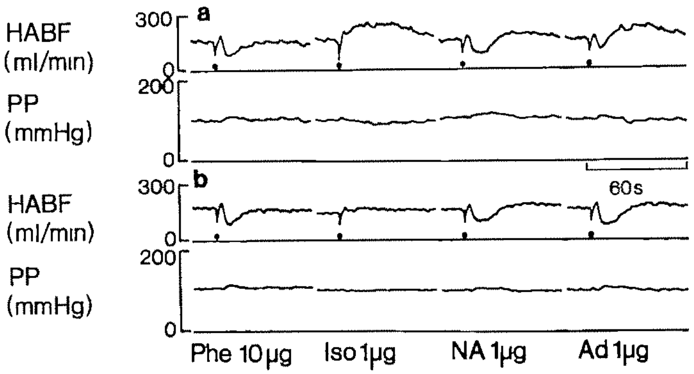

**Figure 1** The effects of intra-arterial injections of phenylephrine (Phe, 10 µg), isoprenaline (Iso, 1.0 µg), noradrenaline (NA, 1.0 µg), and adrenaline (Ad, 1.0 µg) on the hepatic arterial blood flow (HABF) and perfusion pressure (PP), (a) before and (b) after the intravenous administration of propranolol (250 µg/kg).

receptor stimulants. The drugs used were: atenolol (Tenormin, ICI), phentolamine mesylate (Rogitine, Ciba) and propranolol hydrochloride (Inderal, ICI).

Drugs were dissolved in, or diluted from ampoules with saline; fresh solutions were prepared for each experiment.

The volume of the external vascular circuit was compensated with a solution of low molecular weight dextran in saline (Rheomacrodex, Pharmacia).

### *Assessment of drug effects*

Agonists were injected intra-arterially in increasing, graded doses to establish the dose-response relationships, and $\log_{10}$ dose-response curves constructed from these data. Where vasodilatation occurred, the maximum change in calculated HAVR was established, and for comparative purposes the $ED_{50}$, defined as the dose of an agonist which would produce 50% of the established maximum for that agonist, is cited. Where vasoconstriction occurred, the absolute maximum response could not be established without injecting such large doses that pronounced systemic effects would have resulted from the locally-injected agents passing through the liver and entering the systemic circulation. In such cases, for comparative purposes the dose of agonist which produced a doubling in the calculated HAVR (i.e. a 100% increase) is cited.

The influence of antagonists upon the responses to agonists are assessed from dose-response curves obtained in the presence and absence of the antagonists. In addition, the antagonist-induced shift in the $ED_{50}$ where the maximum effect could be established, or the dose-ratio for a doubling in HAVR, i.e. (dose of agonist required to double HAVR in the presence of antagonist)/(dose of agonist required to double HAVR in the absence of antagonist) are cited for comparative purposes.

Except where indicated to the contrary, results are expressed as means ± s.e. mean, and initial control values as means ± 1 s.d. The significance of differences between sets of paired data is assessed using Student's *t*-test.

## Results

### *Control values*

Under control conditions, the systemic arterial mean blood pressure was 132.8 ± 16.6 (mean ± s.d.) mmHg and the inferior vena caval pressure 1.5 ± 0.7 mmHg. The hepatic arterial blood flow (HABF) was 202.9 ± 57.6 ml/min and the hepatic arterial perfusion pressure (PP) 118.6 ± 14.6 mmHg, giving a calculated hepatic arterial vascular resistance (HAVR) of 0.64 ± 0.23 mmHg $ml^{-1}$ minute. The livers weighed 292.2 ± 53.8 g, and expressed per unit weight of the livers, the hepatic arterial blood flow was 70.5 ± 17.3 ml $min^{-1}$ $100^{-1}$ and the HAVR 1.82 ± 0.71 mmHg $ml^{-1}$ min 100 g.

### *Effects of phenylephrine, isoprenaline, noradrenaline and adrenaline on the hepatic arterial vascular bed*

*Phenylephrine.* Phenylephrine was injected in increasing, graded, doses over the dose range 100 ng to 100 µg or 200 µg in each of 5 experiments. At all doses above the threshold, which was 1.0 µg in all 5 experiments, vasoconstriction, manifest as rises in the calculated hepatic arterial vascular resistance, was the only response of the hepatic arterial vasculature. In no experiment was any increase in hepatic arterial blood flow observed to succeed the initial vasoconstrictor effect after any dose of phenylephrine. This effect was of short duration (Figure 1) and was dose-dependent (Figure 2), the maximum rise in HAVR of

384.1 ± 73.4% occurring with doses between 50 and 200 µg; the mean dose required to double the HAVR was $1.32 \times 10^{-7}$ mol (Table 2). The effects of the maximal vasoconstrictor doses of phenylephrine on the hepatic arterial blood flow, perfusion pressure and calculated HAVR are shown in Table 1.

*Isoprenaline.* The responses of the hepatic arterial vasculature to intra-arterial injections of isoprenaline have been described previously (Richardson & Withrington, 1976b, d). In both the present and previous experiments, the only hepatic arterial vascular response resulting from intra-arterial injections of isoprenaline over the dose range 10 ng to 50 µg was a dose-dependent increase in hepatic arterial blood flow at a constant or slightly reduced perfusion pressure (Figure 1). This vasodilatation was of slower onset and longer duration of action than the vasoconstrictor response to intra-arterial phenylephrine (Figure 1). No subsequent vasoconstrictor responses were observed after the intra-arterial injection of isoprenaline in the present experiments; the maximum reduction in the calculated HAVR to isoprenaline was 39.1 ± 2.0% ($n=9$). The effects of maximal vasodilator doses of isoprenaline have been described previously (Richardson & Withrington, 1976d).

*Noradrenaline.* Noradrenaline was injected into the sympathetically-innervated hepatic arterial vascular bed in graded increasing doses over the dose-range 1 ng to 100 µg to construct dose-response curves in 12 experiments. In contrast to the vascular responses to phenylephrine and isoprenaline, the hepatic arterial vascular responses to intra-arterial noradrenaline were, over most of the dose-range employed, biphasic.

An initial dose-dependent reduction in the hepatic arterial blood flow was followed at all but the highest doses of noradrenaline by a delayed and more sustained increase in flow (Figure 1). The consequent initial rise in calculated HAVR, indicating vasoconstriction, and the subsequent fall in calculated HAVR, indicating vasodilatation, were related to the dose of noradrenaline injected (Figure 2) until doses in excess of 10 or 50 µg in different experiments were injected, when no secondary effect was apparent. The

**Table 1** Maximal vasoconstrictor and vasodilator effects of phenylephrine, noradrenaline and adrenaline on hepatic arterial perfusion pressure (PP), hepatic arterial blood flow (HABF) and calculated hepatic arterial vascular resistance (HAVR)

| | *Phenylephrine* | *Noradrenaline* | *Adrenaline* |
|---|---|---|---|
| *Vasoconstrictor responses* | | | |
| Maximal doses (µg) | 50–200 | 50–200 | 50–200 |
| PP control (mmHg) | 108.3 ± 1.7 | 100 2 ± 3.6 | 102.5 ± 6 5 |
| PP peak response (mmHg) | 121.5 ± 2.3 | 121.6 ± 4.0 | 119.9 ± 6.9 |
| HABF control (ml min$^{-1}$ 100 g$^{-1}$) | 70.0 ± 6.2 | 83.6 ± 7.0 | 64.1 ± 5.8 |
| HABF peak response (ml min$^{-1}$ 100 g$^{-1}$) | 17.4 ± 4.4 | 31.6 ± 5.4 | 16 4 ± 3.5 |
| HAVR control (mmHg ml$^{-1}$ min 100 g) | 1.58 ± 0.12 | 1.27 ± 0.12 | 1.74 ± 0.22 |
| HAVR peak response (mmHg ml$^{-1}$ min 100 g) | 8.32 ± 1.80 | 5.81 ± 1.53 | 10.79 ± 1.90 |
| *Vasodilator responses* | | | |
| Maximal doses (µg) | | 1–50 | 0.05–50 |
| PP control (mmHg) | | 101 8 ± 3.1 | 110.5 ± 6.2 |
| PP peak response (mmHg) | | 101.3 ± 3.5 | 106 3 ± 5.7 |
| HABF control (ml min$^{-1}$ 100 g$^{-1}$) | | 78.1 ± 6.0 | 67.7 ± 4.6 |
| HABF peak response (ml min$^{-1}$ 100 g$^{-1}$) | | 92.0 ± 6 3 | 82.6 ± 3 6 |
| HAVR control (mmHg ml$^{-1}$ min 100g) | | 1.36 ± 0 11 | 1.74 ± 0.21 |
| HAVR peak response (mmHg ml$^{-1}$ min 100 g) | | 1.13 ± 0.08 | 1.33 ± 0.13 |

Values are shown as means ± s.e. means for points immediately before maximal vasoconstrictor and vasodilator doses (control) and at the maximum effect (peak response). The data are derived from the same experiments as in Figure 2; comparable values for isoprenaline have been published previously (Richardson & Withrington, 1976d).

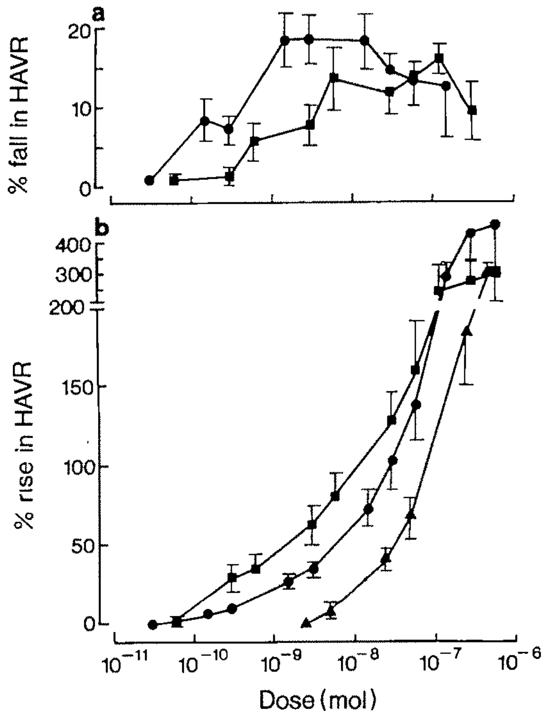

**Figure 2** Dose-response curves to the intra-arterial injection of phenylephrine (▲), noradrenaline (■) and adrenaline (●) Abscissa scale: dose, expressed as a fraction of one mole of each agent injected intra-arterially Ordinate scale: (a) percentage reduction in calculated hepatic arterial vascular resistance (HAVR), (b) percentage increase in HAVR. The symbols represent the means of 5 observations for phenylephrine, 12 for noradrenaline and 13 for adrenaline, and the vertical bars show the s.e. means.

threshold dose for the initial vasoconstriction was between 10 and 100 ng, and in all but one experiment the threshold for the subsequent vasodilator effect was higher (10 ng to 5.0 µg). The time course of the initial vasoconstriction and the subsequent dilatation, characteristic of the response to intra-arterial noradrenaline were similar to the monophasic responses to equipotent doses of phenylephrine and isoprenaline, respectively (Figure 1).

The dose-response curves (Figure 2) reveal that noradrenaline was a more potent vasoconstrictor of the hepatic arterial vasculature than phenylephrine, the mean dose of noradrenaline required to double the HAVR in 5 experiments was $1.34 \times 10^{-8}$ mol, being significantly smaller than the corresponding dose for phenylephrine in the same experiments ($P < 0.025$). The maximum increase in calculated HAVR to noradrenaline was 335.5 ± 74.4%, representing minimum hepatic arterial blood flows which were not significantly different from those attained at maximal vasoconstriction with phenylephrine ($P > 0.20$: for analysis see Richardson & Withrington, 1976a). The maximum secondary reduction in HAVR to noradrenaline of 16.0 ± 2.4% was however significantly smaller than the maximum response to isoprenaline of 38.6 ± 2.9% ($P < 0.001$) where maximum responses to both agents were established in the same 8 preparations.

*Adrenaline.* Adrenaline was injected into the hepatic artery in increasing graded doses over the range 10 ng–100 µg to obtain dose-response curves in 13 experiments. In five experiments, the injections were 'paired' with equal-weight administrations of noradrenaline and phenylephrine, for analysis of the relative potencies of these agents. The hepatic arterial vascular responses to doses of adrenaline above threshold were, as to noradrenaline but in contrast to phenylephrine and isoprenaline, biphasic (Figure 1). An initial reduction in hepatic arterial blood flow of rapid onset and short duration was succeeded at most doses by a more protracted increase in blood flow. The time courses of the two responses were similar to those of noradrenaline (Figure 1), and both effects were dose-dependent (Figure 2), though at the higher doses (5–50 µg) the secondary vasodilatation was masked by the predominant primary vasoconstriction, as with noradrenaline (Figure 2). The threshold vasoconstrictor doses for adrenaline of 10 ng to 500 ng were slightly higher than for noradrenaline, whilst the threshold vasodilator doses were slightly lower (10–100 ng) than for noradrenaline. In individual experiments, the threshold doses for the two effects of adrenaline were, in contrast to noradrenaline, usually coincident. The dose-response curves (Figure 2) reveal that on a molar basis, adrenaline has a potency as a constrictor of the hepatic arterial vascular bed intermediate between noradrenaline and phenylephrine; the mean dose of adrenaline required to double the calculated HAVR ($3.06 \times 10^{-8}$ mol) was significantly smaller ($P < 0.025$) than that for phenylephrine, though not significantly greater ($P > 0.10$) than for noradrenaline in the same 5 experiments. The maximum rise in HAVR of 485.2 ± 75.3% was attained with doses of adrenaline which produced minimum hepatic arterial blood flows not significantly different from those attained with maximum vasoconstrictor doses of noradrenaline ($P > 0.20$) or phenylephrine ($P > 0.10$); details of the maximum vasoconstrictor effects of adrenaline are given in Table 1. It is not, therefore possible on the basis of these experiments to establish any particular order of maximum vasoconstrictor potency for phenylephrine, noradrenaline and adrenaline (see Richardson & Withrington, 1976a).

The maximum vasodilator effect of adrenaline (21.9 ± 2.9% reduction in the calculated HAVR) whilst slightly greater than that for noradrenaline, was significantly less than that for isoprenaline (35.9 ± 1.9%; $P<0.0025$) in the same 10 experiments where maximal responses to both were established. Adrenaline was, however, a more potent hepatic arterial vasodilator than noradrenaline, as revealed by the significantly smaller $ED_{50}$ dose for adrenaline ($P<0.005$) (Table 2).

*Responses of the hepatic arterial vascular bed to intra-arterial injections of phenylephrine, isoprenaline, noradrenaline and adrenaline after the systemic administration of propranolol*

The nonselective β-adrenoceptor antagonist, propranolol, was injected intravenously in a dose previously found adequate to attenuate the positive chronotropic effects of intravenous isoprenaline (5 µg). Propranolol itself caused a transient increase in the calculated hepatic arterial vascular resistance of 13.0 ± 6.4%, when injected intravenously ($P>0.10$, $n=5$).

*Phenylephrine.* The hepatic arterial vascular response to intra-arterial phenylephrine was not significantly modified by pretreatment with propranolol, both the form and position of the dose-response curve to phenylephrine being unaffected (Figure 3). The dose of phenylephrine required to double the calculated HAVR in 3 experiments was 20.9 ± 7.2 µg before propranolol and 14.4 ± 6.0 µg after, the difference between these values being statistically insignificant ($P>0.50$). The dose-ratio for this effect was 0.79 ± 0.28.

*Isoprenaline.* Propranolol greatly attenuated the hepatic arterial vasodilator response to isoprenaline in each of 3 experiments, there being a marked parallel shift of the isoprenaline dose-response curve to the right without suppression of the maximum responses compared with the control. The $ED_{50}$ dose of isoprenaline before propranolol in these 3 experiments was 0.10 ± 0.05 µg and after β-adrenoceptor blockade this was significantly increased to 2.93 ± 0.22 µg ($P<0.05$). After propranolol, isoprenaline did not evoke hepatic arterial vasoconstriction at any dose. However propranolol raised the threshold to isoprenaline, and thereafter doses of isoprenaline overcame the competitive blockade and resulted in hepatic arterial vasodilatation.

*Noradrenaline.* The biphasic response to intra-arterial noradrenaline was modified by pretreatment with propranolol; the secondary vasodilator response was reduced (Figure 1), whilst the initial vasoconstrictor response was slightly increased over most of the dose-range (Figure 3).

The dose-response curve for the vasoconstrictor effect of noradrenaline was shifted to the left by propranolol, though neither the reduction in the dose of noradrenaline required to double the HAVR from 0.86 ± 0.8 µg to 0.69 ± 0.26 µg ($n=3$), representing a dose-ratio for this effect similar to that for

**Table 2** Relative potencies of phenylephrine, isoprenaline, noradrenaline and adrenaline on the hepatic arterial vascular bed of the dog

| *Initial vasoconstrictor responses* | | |
|---|---|---|
| | *Threshold dose (range)* | *Dose (± s.e. mean) required to double HAVR* |
| Phenylephrine | $4.9 \times 10^{-9}$ | $1.32\ (\pm 0.04) \times 10^{-7*\ **}$ |
| Noradrenaline | $5.9 \times 10^{-11}$ to $5.9 \times 10^{-10}$ | $1.34\ (\pm 0.71) \times 10^{-8*}$ |
| Adrenaline | $3.0 \times 10^{-11}$ to $1.5 \times 10^{-9}$ | $3.06\ (\pm 0.10) \times 10^{-8}$ ** |
| *Vasodilator responses* | | |
| | *Threshold dose (range)* | *$ED_{50}$ dose (±s.e. mean)* |
| Isoprenaline | $1.8 \times 10^{-11}$ to $9.0 \times 10^{-11}$ | $2.90\ (\pm 0.90) \times 10^{-10}$ |
| Noradrenaline | $5.9 \times 10^{-11}$ to $3.9 \times 10^{-9}$ | $3.96\ (\pm 0.77) \times 10^{-9***}$ |
| Adrenaline | $3.0 \times 10^{-11}$ to $3.0 \times 10^{-10}$ | $5.52\ (\pm 1.90) \times 10^{-10***}$ |

All doses are expressed as fractions of one mole injected intra-arterially. The ranges for the thresholds are derived from all dose-response curves constructed (phenylephrine = 5, noradrenaline = 12, adrenaline = 13, isoprenaline = 12), and the doses required to double the hepatic arterial vascular resistance (HAVR) and $ED_{50}$ values are derived from 5 experiments in which dose-response curves to all 4 substances were constructed by injecting blocks of the same weight of each substance followed by the next higher dose, again of all four substances in turn. Statistically significant differences between pairs of data obtained in this way are shown by * and ** = $P<0.025$; *** = $P<0.005$. Data included in the isoprenaline range are derived from Richardson & Withrington (1976b).

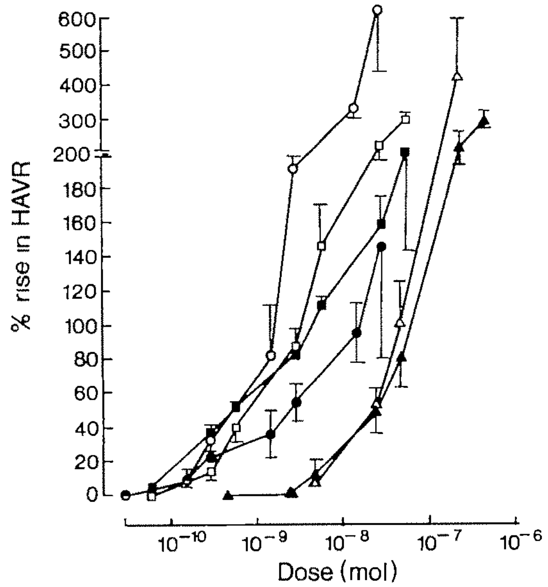

**Figure 3** Dose-response curves for the vasoconstrictor actions of phenylephrine, noradrenaline and adrenaline on the canine hepatic arterial vascular bed. The doses are expressed as a fraction of one mole of each substance injected intra-arterially (abscissa scale), and the responses as the percentage increase in the calculated hepatic arterial vascular resistance (HAVR) (ordinate scale). In all cases, the points represent the means of three observations: noradrenaline before propranolol (■), noradrenaline after propranolol (□); adrenaline before propranolol (●); adrenaline after propranolol (○); phenylephrine before propranolol (▲); phenylephrine after propranolol (△). The vertical bars show the s.e. means.

phenylephrine of 0.78 ± 0.24, nor the increases in the vasoconstrictor effects of individual doses of noradrenaline (Figure 3) were statistically significant ($P > 0.05$). Propranolol pretreatment resulted in a marked suppression of the vasodilator effect of all doses of noradrenaline (Figure 1), suggesting that it was due to β-adrenoceptor stimulation.

*Adrenaline.* As with noradrenaline, the hepatic arterial vascular responses to intra-arterial adrenaline after propranolol were modified over most of the dose-range, the secondary delayed vasodilatation due to adrenaline being markedly attenuated by propranolol (Figure 1). The vasoconstrictor potency of adrenaline was significantly enhanced by propranolol (Figure 3), the dose-response curve being shifted to the left of the control dose-response curve. The dose of adrenaline required to double the HAVR fell significantly ($P < 0.005$) from 11.8 ± 4.4 μg before propranolol to 0.6 ± 0.1 μg after β-adrenoceptor blockade, the dose-ratio for this effect of 0.09 ± 0.06 being very much smaller than the corresponding dose-ratios for noradrenaline and phenylephrine.

Furthermore, after propranolol, the dose-response curve for the vasoconstrictor effect of adrenaline lay to the left of the corresponding curve for noradrenaline in the same three preparations (Figure 3), in contrast to the potency order before propranolol (Figures 2 and 3). After β-adrenoceptor blockade therefore, adrenaline was a more potent hepatic arterial vasoconstrictor than noradrenaline or phenylephrine (Figure 3).

The attenuation of the vasodilator responses to intra-arterial noradrenaline and adrenaline by propranolol (Figure 1) and the consequent potentiation of the hepatic arterial vasoconstrictor responses to these catecholamines, particularly to adrenaline (Figure 3) represents evidence that a component of their normal hepatic arterial vascular actions is mediated through β-adrenoceptors.

### *Nature of the β-adrenoceptors in the hepatic arterial vascular bed of the dog*

β-Adrenoceptors have been demonstrated to be present in the hepatic arterial vasculature of the dog (Richardson & Withrington, 1976b,d) and from the present experiments, appear to contribute to the responses of this vascular bed to intra-arterial injections of the naturally-occurring catecholamines noradrenaline and adrenaline. The nature of the β-adrenoceptor population at this site has been examined further by the use of the selective $\beta_2$-adrenoceptor stimulant, salbutamol (Daly, Farmer & Levy, 1971) and the selective $\beta_1$-adrenoceptor antagonist atenolol (Hainsworth *et al.*, 1974).

*Hepatic arterial vascular responses to isoprenaline and salbutamol.* The complete dose-response curve to isoprenaline on this preparation has been published previously (Richardson & Withrington, 1976d). The dose-response relationship for salbutamol was similarly constructed in 3 experiments by the injection of increasing graded doses over the range 10 ng to 50 μg. All doses of salbutamol above the threshold (50–100 ng) caused only dose-dependent hepatic arterial vasodilatation with the maximum effect, a fall in HAVR of 39.5 ± 2.7%, occurring at 20 or 50 μg (Figure 4). These responses and their time courses were similar to the responses to isoprenaline (Richardson & Withrington, 1976d). In two experiments in which paired equal weight doses of the two agonists were administered it was evident that at each weight injected, the hepatic arterial vasodilator response to isoprenaline was greater than that to salbutamol (Figure 4). The greater intrinsic potency of isoprenaline in stimulating β-adrenoceptors was

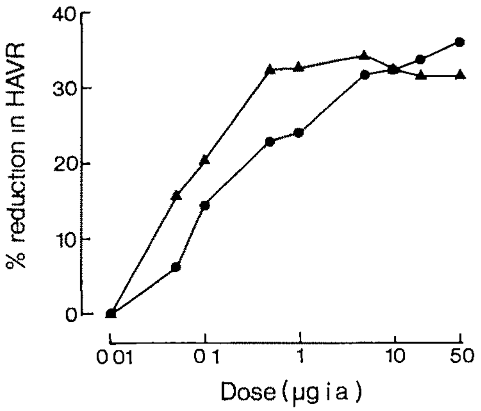

**Figure 4** Log-response curve for isoprenaline (▲) and salbutamol (●) The doses are expressed in terms of the weight of each substance injected intra-arterially in the same experiment (abscissa scale) and the response as the percentage reduction in the calculated hepatic arterial vascular resistance (HAVR) (ordinate scale).

confirmed when the results were expressed on a molar basis where the mean $ED_{50}$ for isoprenaline ($6.3 \times 10^{-10}$ mol) is less than that for salbutamol ($3.5 \times 10^{-9}$ mol).

*Responses of the hepatic arterial vascular bed to isoprenaline in the presence of propranolol or atenolol.* In two experiments, dose-response curves to intra-arterial isoprenaline were constructed (i) in the absence of antagonists (ii) after the intravenous administration of atenolol (100 µg/kg), a dose which almost completely blocked the positive chronotropic responses to isoprenaline (5 µg i.v.) and (iii) after propranolol (100 µg/kg).

The dose-response curves obtained in both of these experiments revealed that atenolol caused neither a shift in the position of the dose-response curve nor any suppression of the maximum vasodilator response. In contrast, the intravenous administration of propranolol caused a marked and parallel shift of the dose-response curve to the right with no suppression of the maximum response; characteristics of competitive antagonism. The results from one such experiment are shown in Figure 5.

The observations that salbutamol, a relatively selective $\beta_2$-adrenoceptor stimulant, evokes marked hepatic arterial vasodilatation and that the responses of the bed to isoprenaline are not antagonized by atenolol, a $\beta_1$-adrenoceptor antagonist, supports the conclusion that the $\beta$-adrenoceptors present in the hepatic arterial vascular bed of the dog are predominantly of the $\beta_2$ type.

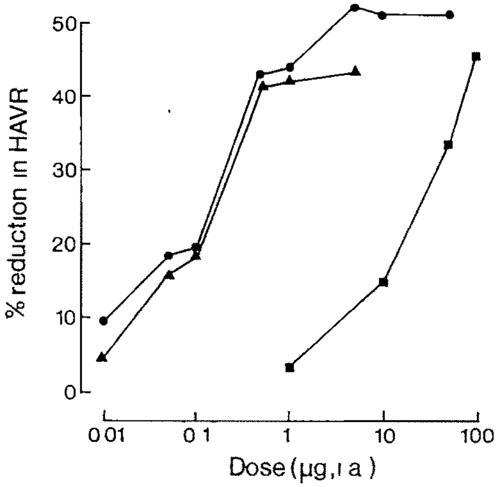

**Figure 5** Effects of isoprenaline injected intra-arterially to the canine liver before (▲) and after atenolol, 100 µg/kg (●) and after propranolol, 100 µg/kg (■) in the same experiment. Doses of isoprenaline are expressed in terms of the weight injected (abscissa scale) and the response as the percentage reduction in the calculated hepatic arterial vascular resistance (HAVR) (ordinate scale). The antagonists were injected intravenously.

### *The role of α-adrenoceptors in the responses of the hepatic arterial vascular bed to intra-arterial phenylephrine, isoprenaline, noradrenaline and adrenaline*

Dose-response curves to phenylephrine, isoprenaline, noradrenaline and adrenaline were constructed in two experiments before and after the administration of the α-adrenoceptor antagonist, phentolamine (500 µg/kg, i.v.). Phentolamine caused a suppression of the hepatic arterial vasoconstrictor actions of phenylephrine, noradrenaline and adrenaline, the dose-response curves to all three being shifted to the right in a parallel manner without a reduction in the maximum response that could be elicited. However, quantitative comparisons of the potencies of the vasoconstrictor agents before and after phentolamine is complicated by the alteration in the control hepatic arterial vascular resistance caused by the administration of phentolamine (Hanson, 1973).

It is not possible to assess the influence of α-adrenoceptor blockade upon the vasodilator responses to isoprenaline or the secondary falls in HAVR due to adrenaline and noradrenaline because the vasodilator action of phentolamine reduced the vascular tone necessary for the quantitative evaluation of vasodilatation in this bed (Richardson & Withrington, 1976d; 1977a).

Despite the difficulty in making quantitative deductions from these experiments, they demonstrate clearly that the vasoconstrictor responses to phenylephrine, noradrenaline and adrenaline injected intra-arterially into the hepatic artery are dependent upon α-adrenoceptor stimulation.

## Discussion

The use of the sympathetically-innervated arterial vascular bed of the dog's liver for the quantitative study of the effects of vasodilator hormones has previously been reported from this laboratory, and the relative molar potencies of a range of naturally-occurring and synthetic vasodilator agents established (Richardson & Withrington, 1976c, d; 1977a). In the present experiments, this preparation has been used to study the vascular effects of noradrenaline and adrenaline, injected intra-arterially, comparing these with the synthetic α- and β-adrenoceptor agonists, phenylephrine and isoprenaline respectively. In this way, the contribution of α- and β-adrenoceptor stimulation to the vascular effects of the naturally-occurring catecholamines on the hepatic arterial vascular bed can be ascertained.

Phenylephrine, injected into the hepatic arterial vasculature, caused reductions in the hepatic arterial blood flow at constant or, with high doses, slightly increased perfusion pressure, changes indicative of hepatic arterial vasoconstriction. These effects were dose-dependent and were not followed at any dose level in any experiment by secondary rises in hepatic arterial blood flow, which would indicate hepatic arterial vasodilatation. It is established that phenylephrine produces vasoconstriction in other vascular beds by direct α-adrenoceptor stimulation, and is devoid of actions on cardiac and other β-adrenoceptors (Aviado, 1959; 1970; Innes & Nickerson, 1975): on these grounds, it is commonly used as a pure α-adrenoceptor stimulant in the analysis and definition of adrenoceptor populations in vascular and non-vascular tissues. In the present experiments the specificity of phenylephrine as an α-adrenoceptor agonist was confirmed by the lack of antagonism by propranolol: a dose of propranolol sufficient to cause a shift of the isoprenaline dose-response curve on the hepatic arterial vasculature to the right, was without any effect on the form or position of the phenylephrine dose-response curve in this vascular bed. Consequently, the response to intra-arterial injections of phenylephrine is due solely to α-adrenoceptor stimulation in the canine hepatic arterial vasculature; this always caused an increase in hepatic arterial vascular resistance.

Isoprenaline, the archetypal β-adrenoceptor stimulant, has previously been shown to produce dose-dependent hepatic arterial vasodilatation (Richardson & Withrington, 1976b, d) manifest as an increase in hepatic arterial blood flow at constant, or with high doses, slightly reduced perfusion pressure. The systemic administration of propranolol caused a parallel shift of the isoprenaline dose-response curve to the right without suppression of the maximum response, but did not reveal a vasoconstrictor action of isoprenaline due to α-adrenoceptor stimulation: after propranolol, high doses of isoprenaline overcame the competitive β-blockade (Figure 4) without revealing any additional effects of the agonist. The responses of the hepatic arterial vascular bed to intra-arterial isoprenaline are therefore taken to represent pure β-adrenoceptor stimulation, an action resulting in a reduction in hepatic arterial vascular resistance.

The actions of the two naturally-occurring catecholamines, noradrenaline and adrenaline, on the hepatic arterial vascular bed, are more complex than those of phenylephrine and isoprenaline: over most of the dose-ranges used, intra-arterial injections of noradrenaline and adrenaline resulted in biphasic effects. Typically, an initial vasoconstriction of rapid onset and short duration was succeeded by a vasodilatation of longer duration, both effects being dose-dependent (Figure 2). Similar vascular effects have been noted previously (Andrews *et al.*, 1955). When equal weights of the two agents were injected, the vasoconstrictor response was of greater magnitude with noradrenaline, and the vasodilator response greater with adrenaline. In the absence of antagonists, the vasoconstrictor potency of noradrenaline was greater than that of adrenaline, and both were significantly more potent hepatic arterial vasoconstrictors than phenylephrine (Figure 2, Table 2); the maximal vasoconstriction attained in these experiments was similar with all three substances, and on the basis of these experiments, they cannot be ranked in any particular order of maximal vasoconstrictor potency. Quantitative comparisons of the vasodilator responses are however rather more difficult since with high doses of both noradrenaline and adrenaline, the secondary vasodilatation was no longer apparent, being masked by the substantial primary vasoconstrictor response. The maximum vasodilator responses to noradrenaline and adrenaline could not therefore be established with certainty. Adrenaline was a more potent hepatic arterial vasodilator than noradrenaline, the molar dose-response curve for the vasodilator action of adrenaline being to the left of the corresponding curve for noradrenaline (Figure 2); further, the $ED_{50}$ for the vasodilator action of adrenaline was smaller than that for noradrenaline, but since the maximum effects may not have been established, this may be a less reliable means of comparison of potencies: the maximum reduction in hepatic arterial vascular resistance with both noradrenaline and adrenaline was significantly smaller than that with isoprenaline in the same experiments.

It is not practicable in this preparation to assess these vasodilator effects in a more rigidly controlled manner, since the administration of $\alpha$-adrenoceptor antagonists, which might be expected to uncover maximal vasodilator effects by abolishing the primary vasoconstrictor responses to the catecholamines, *a priori* diminishes the hepatic arterial sympathetic vasoconstrictor tone and results in an alteration in the baseline vascular resistance, making quantitative comparisons difficult.

The systemic administration of propranolol, an established nonselective $\beta$-adrenoceptor antagonist was, in contrast to $\alpha$-adrenoceptor blockade, without sustained effect on the hepatic arterial vascular resistance (see Hanson, 1973). Propranolol competitively antagonized the effects of isoprenaline on this vascular bed, and was without effect on the responses to phenylephrine. It attenuated the secondary vasodilator responses to noradrenaline and adrenaline (Figure 1) and increased the vasoconstrictor responses to adrenaline. The enhancement of the vasoconstrictor effects of adrenaline was particularly marked, and was demonstrated to be statistically significant: after propranolol, adrenaline was, on a molar basis, a more potent hepatic arterial vasoconstrictor than noradrenaline, the reverse of the potency order in the absence of antagonists, and the same as the potency order found in the hepatic portal venous vascular bed in the absence of antagonists (Richardson & Withrington, 1977b).

This attenuation by propranolol of the vasodilator responses to noradrenaline and adrenaline, and the potentiation of the vasoconstrictor responses to adrenaline injected intra-arterially to the liver unequivocally demonstrate the involvement of $\beta$-adrenoceptors in the reponses to these catecholamines. This conclusion accords with the results of Greenway & Lawson (1969) in the cat, but is contrary to the views of Green *et al.* (1959) using the dog, who concluded that only $\alpha$-adrenoceptors are present in hepatic arterial resistance vessels.

The conclusion from these observations is that the initial vasoconstrictor response to noradrenaline and adrenaline is due to $\alpha$-adrenoceptor stimulation, and the succeeding vasodilatation is due to $\beta$-adrenoceptor stimulation. The effects of the four catecholamines studied therefore depend directly upon their relative efficacies as $\alpha$- and $\beta$-adrenoceptor stimulants. It is improbable that the secondary vasodilatation arises as a consequence of the primary vasoconstriction whether mediated by local or systemic mechanisms, since: (i) phenylephrine, which exerted as great a vasoconstrictor effect as the catecholamines noradrenaline and adrenaline did not exhibit secondary vasodilatation; (ii) the systemic effects of all substances were small, and similar for each, probably reflecting the deactivation of the substances in passage through the liver and cardiopulmonary circuit (Vane, 1969); (iii) the larger vasoconstrictor effects were not associated with correspondingly larger secondary vasodilator effects as might be expected in a local 'reactive hyperaemia' response.

The hepatic arterial vasculature dilates in response to intra-arterial injections of both salbutamol and isoprenaline, the dose-response curves being parallel and the maximum responses very similar (Figure 4). On a molar basis salbutamol was 18 times less potent than isoprenaline (comparing $ED_{50}$ values), a value similar to that for the effects on arterial pressure, but in contrast to the value (285–792) for the effects on heart rate after intravenous injection (Daly *et al.*, 1971) in the dog. Conversely, the responses of this vasculature to intra-arterial isoprenaline were not antagonized by a dose of atenolol adequate to suppress the positive chronotropic effects of intravenous isoprenaline. Atenolol is cardioselective in animals (Barrett, Carter, Fitzgerald, Hull & Le Count, 1973; Hainsworth *et al.*, 1974; Harry, Knapp & Linden, 1974) and man (Graham, Littlejohns, Pritchard, Scales & Southorn, 1973; Marlin, Kumana, Kaye, Smith & Turner, 1975), and its lack of effect in the present experiments suggests that if there are $\beta_1$-adrenoceptors in this vascular bed, their population is small or they are resistance to blockade. The conclusion from these studies is that the predominant $\beta$-adrenoceptor population in the hepatic arterial vascular bed of the dog is of the $\beta_2$ type.

The differences in the time courses of the responses to $\alpha$- and $\beta$-adrenoceptor stimulation prompt the question of the possible locations of these receptors. One established physiological action of adrenaline is to stimulate liver parenchymal metabolism by the activation of adenylcyclase (Exton, Mallette, Jefferson, Wong, Friedman & Park, 1970); these metabolic changes, classically described as a $\beta$-action of adrenaline (Newton & Hornbrook, 1972), may alter the vascular smooth muscle environment or activity to cause a delayed and prolonged vasodilatation. Whilst it may be that the hepatic arterial vasodilatation is a secondary effect of $\beta$-adrenoceptor-mediated stimulation of liver metabolism, the two effects, both mediated by $\beta$-adrenoceptors may well have distinct receptor sites. It seems probable that the vasodilator action of adrenaline is of physiological importance in ensuring an adequate blood flow to the hepatic parenchyma in states of stimulated liver metabolism; in this context, the study of the effects of intra-arterial infusions of catecholamines which may more closely mimic the physiological release of suprarenal medullary catecholamines than acute injections, may be of importance, as may the possible interactions between adrenaline and other hormones such as glucagon (Richardson & Withrington, 1976a, c; 1977a).

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# THE EFFECTS OF PROSTAGLANDIN $E_1$, BRADYKININ AND HISTAMINE ON CANINE SYNOVIAL VASCULAR PERMEABILITY

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1 The relative effects of prostaglandin $E_1$, bradykinin and histamine on canine synovial vascular permeability were investigated by a method based on the measurement of the amounts of radiolabelled dextran (molecular weight 20,000) leaking from the circulation into the synovial cavity.

2 Bradykinin, prostaglandin $E_1$ and histamine in that order of potency all increased synovial vascular permeability. Threshold concentrations were 0.3 μM, 3 μM and 30 μM respectively.

3 The effects of infusion of prostaglandin $E_1$ combined with either bradykinin or histamine were greater than mere summation.

## Introduction

Prostaglandins and kinins have been demonstrated in increased amounts in the synovial fluid of various arthritides (Melmon, Webster, Goldfinger & Seegmiller, 1967; Higgs, Vane, Hart & Wojtulewski, 1974) and together with histamine may play a part in human clinical joint disease (Zeitlin & Grennan, 1976). We have previously shown that normal synovial blood flow is extremely sensitive to the vasodilator effects of prostaglandin $E_1$ and bradykinin, and reactive but to a lesser degree, to histamine (Dick, Grennan & Zeitlin, 1976).

In the present study the effects of synovial infusion of these mediators on synovial vascular permeability have been examined by a method based on the exudation of a radiolabelled dextran marker from the circulation into the synovial cavity.

## Methods

### *Animals*

These studies were carried out on adult mongrel dogs weighing between 20 and 30 kg, and anaesthetized with thiopentone, nitrous oxide, oxygen and less than 1% halothane. Blood pressure and blood gases were monitored and maintained constant throughout all experiments.

### *Experimental procedure*

Following intravenous injection of $^{131}$I-labelled dextran dissolved in 0.1 ml of 0.9% w/v NaCl solution (saline), blood samples were taken at 5 min intervals from an in-dwelling catheter lying in the cubital artery. Samples of blood (1 ml) were subsequently counted in a Packard automatic gamma spectrometer. The stifle joint was perfused with sterile normal saline or with the test drug dissolved in saline, via a 25 gauge inflow needle inserted distal to the patella. The perfusing solution passed to the inflow needle via a plastic catheter coiled in a water bath kept at 37°C. The joint was drained continuously through a 19-gauge outflow needle placed distal to the patella with the hind limb arranged so that this needle was dependent and the perfusate could be collected. Flow rate was at 1 ml per min and the needles were arranged so that a free flow of fluid was obtained and the joint was not distended. Positioning of the needles was checked initially by aspiration of synovial fluid from the joint. Perfusion of the joint with saline or test solution was carried out after intravenous injection of radiolabelled dextran and 5 ml aliquots of perfusate were collected at the

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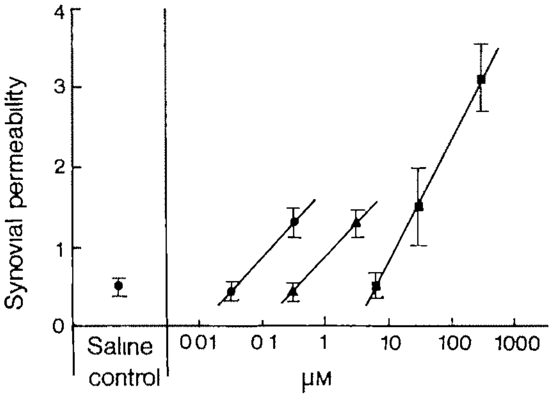

**Figure 1** Dose-response relationships of the permeability increasing actions of bradykinin (●), prostaglandin $E_1$ (▲) and histamine (■) on dog synovial vasculature. Vertical lines show s.e. mean. Permeability to dextran 20 is expressed as· (counts $min^{-1}$ $ml^{-1}$ synovial perfusate) × 100/(counts $min^{-1}$ $ml^{-1}$ blood).

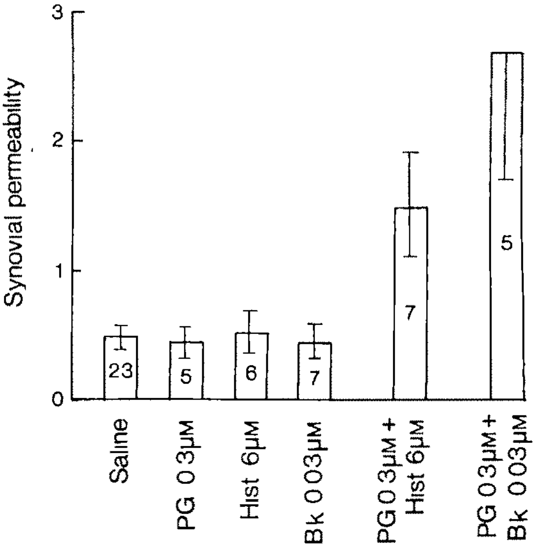

**Figure 2** Interactions between the effects of threshold concentrations of prostaglandin $E_1$ (PG) and histamine (Hist) or bradykinin (Bk) on the permeability of dog synovial vasculature to dextran 20. Vertical lines show s.e. mean. Numbers of joints tested are shown. Permeability is expressed as· (counts $min^{-1}$ $ml^{-1}$ synovial perfusate) × 100/(counts $min^{-1}$ $ml^{-1}$ blood).

same time as blood samples every 5 minutes. If the fluid collected was blood-stained or cloudy the results for that particular joint were discarded. All samples were subsequently counted for 1 min in the Packard automatic gamma spectrometer and the radioactivity per ml of the isotope in the collected perfusate was calculated and expressed as a fraction of the isotope in the blood sample taken at the same time. The effects of various concentrations of mediators in the test joint were compared with those of saline in the control joint perfused for the same length of time and the dose-response relationships of the mediators were established.

The interactions of prostaglandin $E_1$ with bradykinin and histamine were investigated by perfusion of the joint with a sub-threshold concentration of either histamine or bradykinin in the presence and absence of sub-threshold concentrations of prostaglandin $E_1$. The opposite joints in these experiments were used as controls and were perfused with one of the mediators alone or with saline.

Statistical significance of alterations in response was determined using the Mann-Whitney U test. All doses were repeated on from 5 to 7 joints. The saline control was repeated on 23 joints.

### *Dextran*

Dextran 20 (average molecular weight 20,000) was supplied by Pharmacia Fine Chemicals and labelled with $^{131}I$ using chloramine T and sodium metabisulphite. Radiolabelled dextran solutions were made up freshly before each experiment. An aliquot was retained after labelling and on completion of the day's experiment the stability of the label was checked by electrophoresis and by thin layer chromatography using a cellulose plate and 80% ethanol in water as solvent. A single peak was always obtained with both methods showing that the label had been stable for the duration of the experiments.

The effective diffusion radius of dextran 20 is approximately equivalent to that of serum albumin (Grotte, 1956).

### *Drugs*

Histamine was supplied as histamine acid phosphate (Evans Medical). Synthetic bradykinin was obtained from Sandoz Ltd. Prostaglandin $E_1$ (Upjohn Co.) was stored in ethanolic solution at −20°C. Solutions of the various concentrations of drugs tested were made up freshly in saline before each experiment.

## Results

Bradykinin, prostaglandin $E_1$ and histamine all caused increases in synovial permeability. The dose-response relationships are shown in Figure 1. The effects of perfusion with 6 µM histamine were similar to those of the saline control ($P > 0.05$; $n = 6$) while the two higher concentrations 30 μM and 300 μM produced dose-related responses ($P < 0.05$; $n = 4$; $P < 0.001$; $n = 7$, respectively).

Responses to 0.3 μM solutions of prostaglandin $E_1$ were no greater than those to saline ($P > 0.05$; $n = 5$) while 3 μM solutions produced significant effects ($P < 0.001$; $n = 5$). Bradykinin in 30 nM solutions produced no significant effect ($P > 0.05$; $n = 7$) when compared to the saline response while 0.3 μM bradykinin significantly increased permeability ($P < 0.05$; $n = 4$).

The approximate threshold concentrations for these mediators in descending order of potency are bradykinin 0.3 μM, prostaglandin $E_1$ 3 μM and histamine 30 μM.

Figure 2 shows the effects on synovial permeability of sub-threshold concentrations of either histamine or bradykinin when given together with a sub-threshold concentration of prostaglandin $E_1$. The effects both of histamine with prostaglandin $E_1$ ($n = 7$) and of bradykinin with prostaglandin $E_1$ ($n = 5$) appear to be greater than of either drug given alone ($P < 0.025$; $P < 0.001$ respectively). The effects of the two drug combinations were greater than the summation of the effects of the individual drugs by 56% and 167% respectively.

## Discussion

Increased synovial vascular permeability with a resultant increase in synovial fluid volume is a feature of inflammatory joint disease (Boyle & Buchanan, 1971). In rheumatoid arthritis, synovial fluid has been shown to contain raised levels of bradykinin (Melmon *et al.*, 1967; Keele & Eisen, 1970) and prostaglandin (Higgs *et al.*, 1974). Mast cells are plentiful in the rheumatoid synovium (Norton & Ziff, 1966) and histamine and histidine-decarboxylase activity have been detected in the rheumatoid synovial membrane (Roth, Polley & Code, 1964).

Bradykinin (Sturmer & Cerletti, 1961; Lewis, 1964), prostaglandin $E_1$ (Kaley & Weiner, 1971) and histamine (Rocha e Silva, 1966) all increase vascular permeability in a variety of tissues and species and have now been shown to do so in the dog synovium. The most potent of these was bradykinin which increased synovial vascular permeability at an approximate threshold concentration of 0.3 μM. Bradykinin was 10 times more potent on a molar basis than prostaglandin $E_1$. This contrasts with the order of potency of these two mediators on dog synovial blood flow where the prostaglandin was nearly 4 times more active than bradykinin (Dick *et al.*, 1976). In the blood flow study, the drugs tested were administered as single injections into the synovium. The reversal of the potency order might possibly result from the greater lability of bradykinin in contact with biological fluids. This would not be an important factor in the present synovial perfusion method where the synovial level of bradykinin is kept constant.

Ferreira, Moncada & Vane (1974) have shown that the pain producing activity of bradykinin in the dog knee joint is potentiated by infusion of prostaglandins $E_1$ and $E_2$. In the experiments described here, the permeability response to synovial infusion of prostaglandin $E_1$ together with bradykinin or histamine also appears to be greater than a simple summation of effects. Prostaglandin $E_1$ has been shown to potentiate the rat paw oedema produced by bradykinin (Moncada, Ferreira & Vane, 1973; Lewis, Nelson & Sugrue, 1975) and to potentiate the actions of bradykinin and histamine in increasing cutaneous vascular permeability in guinea-pigs (Williams & Morley, 1973). No such potentiation was found in our earlier studies on dog synovial blood flow (Dick *et al.*, 1976).

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# THE USE OF FUNCTIONAL ANTAGONISM TO DETERMINE WHETHER β-ADRENOCEPTOR AGONISTS MUST HAVE A LOWER EFFICACY THAN ISOPRENALINE TO BE TRACHEA-ATRIA SELECTIVE *in vitro* IN GUINEA-PIGS

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1 The relative efficacies of three trachea-atria selective β-adrenoceptor agonists, fenoterol, Me 506 and Me 454, compared to isoprenaline, were determined on both trachea and atria of guinea-pigs.

2 On tracheal preparations the β-adrenoceptor agonists were used as functional antagonists of carbachol and a comparison of the maximum shifts in the carbachol concentration-response line produced by each of the β-adrenoceptor agonists provided a comparison of their efficacies.

3 On atrial preparations carbachol was used as a functional antagonist of the β-adrenoceptor agonists and comparison of the maximum responses to the β-adrenoceptor agonists in the presence of carbachol provided a comparison of their efficacies.

4 On trachea and atria the order of efficacy of the compounds was Me 454 > Me 506 ⩾ isoprenaline = fenoterol.

5 The results indicated that high efficacy in a non-catechol β-adrenoceptor agonist is possible provided there is a favourable N-substituent group.

6 Since Me 454, Me 506 and fenoterol, which are trachea-atria selective, have efficacies equal to or greater than that of isoprenaline, which is non-selective, it is concluded that low efficacy in a compound is not essential for it to show trachea-atria selectivity *in vitro* in guinea-pigs.

## Introduction

A number of β-adrenoceptor stimulants used clinically as bronchodilators produce bronchodilatation without cardiac stimulation, i.e. unlike isoprenaline they are selective. This selectivity of action can be demonstrated *in vitro* in pharmacological experiments in that higher concentrations of selective β-adrenoceptor stimulants are required to give responses on guinea-pig atria than on trachea, whereas this is not so for isoprenaline (O'Donnell & Wanstall, 1974). Lands, Luduena & Buzzo (1967) have proposed that various tissues contain different subtypes of β-adrenoceptor and the conventional view has been that potency differences observed for selective drugs in *in vitro* experiments reflect their affinities for two different receptors in trachea ($\beta_2$) and atria ($\beta_1$). This view has been challenged (Buckner & Abel, 1974; Buckner & Saini, 1975) as a result of data obtained with soterenol. The potency ($EC_{50}$ values) of soterenol was shown to be different on trachea and atria of guinea-pig by Buckner & Abel (1974). However, $EC_{50}$ values are not necessarily a true measure of the affinity of β-adrenoceptor agonists for the receptor (Offermeier, Dreyer & Brandt, 1972; Buckner & Saini, 1975) and from subsequent experiments Buckner & Saini (1975) concluded that soterenol had the same affinity for the receptors in trachea and atria in guinea-pig. Buckner & Saini (1975) suggested that this supported the postulate that the β-adrenoceptors in these two tissues could be of a single type, but they also made two other important points. The first was that the intrinsic activity of soterenol was less than that of isoprenaline on both trachea and atria. Although Buckner & Saini (1975) used the term intrinsic activity, the term efficacy (Stephenson, 1956) has been used in this paper. The term efficacy is equivalent to the term intrinsic activity (Furchgott, 1966). Secondly, they made the statement that the β-adrenoceptor reserve in guinea-pig trachea was approximately 20-fold greater than that in atria, although the supporting data were not at that time published.

It thus appeared that the selectivity of soterenol could not be explained in terms of different affinities for receptors but that it might result from the low efficacy of soterenol if the receptor reserve in guinea-pig trachea was greater than that in the atria. A number of other selective $\beta$-adrenoceptor stimulants, e.g. salbutamol, terbutaline and carbuterol, have been shown to be partial agonists in some tissues, indicating that they have low efficacies, and Jenkinson (1973) envisaged that 'in general, selective $\beta$-agonists may have lower efficacies on all $\beta$-receptors than unselective compounds such as isoprenaline'. The present experiments were therefore undertaken in guinea-pig to determine whether trachea-atria selectivity could occur *in vitro* even with compounds with high efficacy. The compounds selected for study were trachea-atria selective but, although they were all full agonists, their efficacies relative to that of isoprenaline were not known.

A preliminary account of this work was given to the Australian Physiological and Pharmacological Society (O'Donnell & Wanstall, 1976b).

## Methods

Female guinea-pigs (300–550 g) pretreated with reserpine (5 mg/kg, i.p., 24 h previously) were used throughout this study. All concentration-response lines were determined in the presence of metanephrine (50 µM) and phentolamine (10 µM), to block extraneuronal uptake and $\alpha$-adrenoceptors respectively. These drugs were in contact with the tissue for 30 min before each concentration-response line was obtained.

### *Experiments on trachea*

Tracheal chain preparations were set up for isotonic recording as described by O'Donnell & Wanstall (1974). The resting tension on the tissues was 500 mg. Concentration-response lines to carbachol were obtained by the cumulative method of drug addition.

The method used on trachea to obtain efficacy (intrinsic activity) values of the compounds was that advocated by Van den Brink (1973b). A control concentration-response line to carbachol was obtained, followed by further concentration-response lines to carbachol in the presence of different and increasing concentrations of a $\beta$-adrenoceptor agonist. The $\beta$-adrenoceptor agonist was in contact with the tissue for 15 min before obtaining the next carbachol line. The maximum response to carbachol was established at the end of every line. Each response was expressed as a percentage of the maximum contraction which was calculated from the difference between the resting state at the beginning of the line in question and the maximally contracted state at the end of the control line, i.e. in the absence of the $\beta$-adrenoceptor agonist drug. Values for % maximum contraction were plotted against log concentration carbachol; from these graphs the effect of the $\beta$-adrenoceptor agonist drug on the maximum response to carbachol could be ascertained.

From each line the concentration of carbachol producing 50% of the maximum response in that particular line was also interpolated and converted to a negative log value (neg log $EC_{50}$). The $\beta$-adrenoceptor agonist, acting as a functional antagonist, shifted the carbachol line to a higher concentration range (i.e. decreased the neg log $EC_{50}$). For each concentration of $\beta$-adrenoceptor agonist, the difference between the neg log $EC_{50}$ in the absence (control) and presence (functional antagonism) of the $\beta$-adrenoceptor agonist was obtained. This difference is referred to as the log unit shift. Mean values of log unit shift for each concentration of $\beta$-adrenoceptor agonist were obtained from several preparations. In some experiments, after a certain concentration of $\beta$-adrenoceptor agonist, no further decrease in the neg log $EC_{50}$ value for carbachol occurred despite increasing the concentration of $\beta$-adrenoceptor agonist, i.e. a maximum value for log unit shift could be obtained (max. log unit shift). Comparison of the values for max. log unit shift for different $\beta$-adrenoceptor agonists should provide a comparison of their efficacies (Van den Brink, 1973b).

### *Experiments on atria*

Preparations of spontaneously beating atria were set up at 37°C and atrial rate recorded as described by O'Donnell & Wanstall (1974). On these preparations cumulative concentration-response (increase in rate) lines to the $\beta$-adrenoceptor agonists were obtained in the presence of carbachol as a functional antagonist. The maximum rate of beating of the preparation was routinely obtained at the beginning of each experiment by adding a supramaximal concentration of isoprenaline (1 µM). When resting rate was restored, a concentration of carbachol was added which was sufficient to cause a depression in the maximum response to the $\beta$-adrenoceptor agonist being tested. This ranged from 100nM to 500 nM in different experiments. Carbachol decreased the resting atrial rate and this new rate was taken as the baseline rate for the next concentration-response line to $\beta$-adrenoceptor agonist. The difference between this baseline rate and the maximum rate of beating of the preparation was taken as 100% response and all other responses expressed as a percentage of that difference in order to plot log concentration-% maximum response lines.

Up to 3 different $\beta$-adrenoceptor agonists could be compared on each preparation in the presence of the same concentration of carbachol. Comparison of the % maximum responses was used to compare the efficacies of the $\beta$-adrenoceptor agonists.

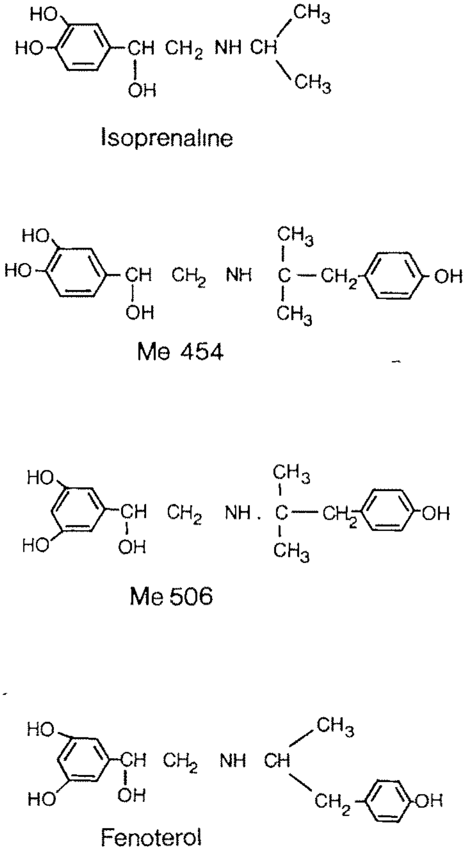

**Figure 1** Structures of isoprenaline, Me 454, Me 506 and fenoterol.

### *Drugs*

The *β*-adrenoceptor agonists used were: fenoterol hydrobromide (Th 1165a, Boehringer-Ingelheim); (±)-isoprenaline sulphate (Burroughs Wellcome); Me 454 base (Boehringer-Ingelheim) and Me 506 hydrobromide (Boehringer-Ingelheim). The structures of these drugs are shown in Figure 1. Other drugs used were: carbachol (Sigma); hydrocortisone hemisuccinate sodium (Glaxo); metanephrine hydrochloride (Calbiochem); phentolamine methanesulphonate (Regitine, Ciba); propranolol hydrochloride (ICI); tropolone (Regis).

All drugs were used as pure powders. The *β*-adrenoceptor agonists were made up in 0.01 N HCl to give stock solutions of 10 or 100 mM. Dilutions were made in Krebs solution containing ascorbic acid (0.2 μg/ml) and kept on ice for the duration of the experiment.

### *Statistical analyses*

The measure of the variation of the mean quoted is the standard error (s.e. mean).

## Results

### *Selection of experimental conditions*

In previous studies in tracheal preparations with intrinsic tone (O'Donnell & Wanstall, 1976a) phenoxybenzamine was used as the inhibitor of extraneuronal uptake. In the present study phenoxybenzamine was unsuitable because it antagonized the contractions to carbachol on trachea. Phenoxybenzamine has been shown to have antimuscarinic activity (Goodman & Gilman, 1975). Both metanephrine (50 μM) and hydrocortisone (50 μM) potentiated, by 2 to 2.5-fold, the responses to isoprenaline on tracheae contracted by 5 μM carbachol. However, the hydrocortisone tended to reduce the maximum contraction that could be attained with carbachol whereas metanephrine did not. The potentiation of isoprenaline produced by 200 μM metanephrine was no greater than that produced by 50 μM. Also, although tropolone (100 μM), a catechol-*O*-methyl transferase inhibitor, caused a 2.5-fold potentiation of isoprenaline, it caused no further potentiation after metanephrine (50 μM). Therefore metanephrine (50 μM) was selected as an inhibitor drug of extraneuronal uptake for inclusion in the Krebs solution. Stimulation of *α*-adrenoceptors was prevented by the inclusion of phentolamine (10 μM) in the Krebs solution as used by Buckner & Saini (1975). This concentration caused little change in the sensitivity of the tracheal preparations to isoprenaline. On atria, inclusion of metanephrine (50 μM) and phentolamine (10 μM) produced no marked change in sensitivity to isoprenaline but these drugs were routinely used to ensure identical experimental conditions for tracheal and atrial preparations. The potency of all the *β*-adrenoceptor agonists in the present study had been shown to be unaffected by cocaine (O'Donnell & Wanstall, 1974). Thus it was not considered necessary to include cocaine in the Krebs solution.

On trachea the values for neg log $EC_{50}$ carbachol obtained using cumulative and single dose additions of the drug were not significantly different. Thus cumulative drug additions were selected. The sensitivity of tracheal preparations to carbachol did not change with repeated lines during the course of an experiment, either in the absence or in the presence of isoprenaline (50 μM), the latter situation necessitating the use of much higher concentrations of carbachol because of functional antagonism. Therefore several concentration-response lines to carbachol could be

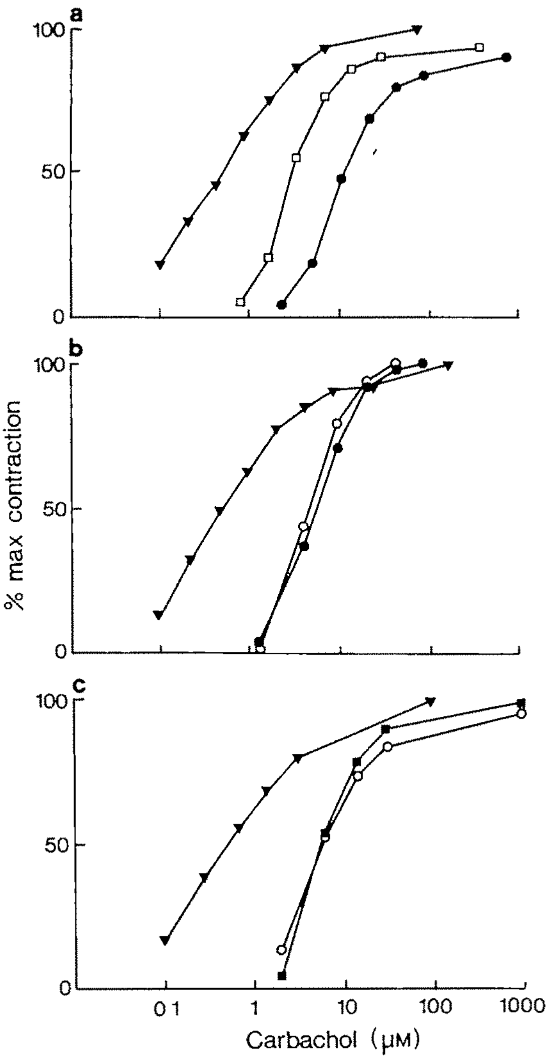

**Figure 2** Functional antagonism of carbachol by isoprenaline in 3 preparations of guinea-pig isolated trachea. Concentration-response lines (with response representing % maximum contraction) to carbachol are shown in the absence (▼) and in the presence of isoprenaline 100 nM (□), 1 μM (●), 5 μM (O) and 20 μM (■). This illustrates that the maximum log unit shift in the carbachol line has been reached in experiments (b) and (c) but not in (a).

obtained on each preparation and no allowances were necessary, in the calculations, for changes in sensitivity.

*Relative efficacy values on trachea*

In the presence of increasing concentrations of isoprenaline, fenoterol, Me 454 or Me 506 there was an increase in the $EC_{50}$ values for carbachol (i.e. a decrease in the neg log $EC_{50}$ values, Table 1) until, where possible, a limiting value was reached. This is illustrated for isoprenaline in Figure 2. Isoprenaline (100 nM and 1 μM) produced progressive shifts in the carbachol concentration-response line (Figure 2a) but no further shift occurred with 5 or 20 μM isoprenaline (Figure 2b and c). Mean values ± s.e. mean of the log unit shift for isoprenaline were: 1.14 ± 0.05 (4), 1.13 ± 0.07 (15) and 1.08 ± 0.05 (26) at concentrations of 1, 5 and 20 μM respectively, with numbers of determinations in parentheses. Thus, in subsequent experiments with other β-adrenoceptor agonists either 5 μM or 20 μM isoprenaline was used to obtain a value for the maximum shift to isoprenaline.

Fenoterol (100 nM, 1 μM and 10 μM) produced progressive shifts in the carbachol line (Figure 3a) with the maximum shift occurring after both 10 and 100 μM fenoterol (Figure 3b, Tables 1 and 2). The maximum log unit shift produced by fenoterol was not significantly different from that produced by isoprenaline (Table 2). Both Me 454 and Me 506 in various concentrations caused a shift in the carbachol line (Figure 3c and d) but it was not possible to establish the maximum shift for either of these drugs. This was because, at concentrations above 100 μM Me 454 and above 1 mM Me 506, any further shift in the carbachol line was accompanied by a depression in the carbachol maximum response (39%–58% depression for Me 454 and 44% depression for Me 506). This was in contrast to fenoterol and isoprenaline for which no marked depression of the maximum response occurred with concentrations which maximally shifted the carbachol line (Table 1). Nevertheless, at 100 μM Me 454 and 1 mM Me 506 the shift produced by the agonist was already significantly greater than the maximum shift produced by isoprenaline in the same experiments (Table 2). Therefore the order of efficacies of the compounds was Me 454 > Me 506 > isoprenaline = fenoterol.

The shifts in the carbachol concentration-response line produced by isoprenaline, fenoterol, Me 454 and Me 506 were prevented if propranolol (1 μM) was present in the Krebs solution.

*Relative efficacy values on atria*

On atria, in the presence of a carbachol concentration which depressed the maximum response to isoprenaline and the other β-adrenoceptor agonists, the maximum response produced by Me 454 was significantly greater than that produced by isoprenaline. The maximum responses to fenoterol and Me 506 were not significantly different from that produced by isoprenaline (Figure 4). The order of efficacies of the compounds was Me 454 > Me 506 ≥ isoprenaline = fenoterol.

## Discussion

At present there is no established irreversible antagonist at β-adrenoceptors available for use in studies on the relative efficacy values of β-adrenoceptor agonists. Therefore in the present study the experimental procedure used by Besse & Furchgott (1976) for estimating the relative efficacies of different agonists on α-adrenoceptors could not be used since it involved the analysis of concentration-response data before and after irreversible inactivation of a fraction of the total receptors. As an alternative procedure, functional antagonism may be used, the theoretical models for which have been put forward by Van den Brink (1973a) and verified by him for β-adrenoceptor agonists (Van den Brink, 1973b). Buckner & Saini (1975) elaborated on the principle of functional antagonism and used it to determine affinity values for β-adrenoceptor agonists in guinea-pig trachea.

In the present study functional antagonism has been used to estimate the relative efficacies for β-adrenoceptor agonists on both trachea and atria of guinea-pig. Van den Brink (1973b) described two experimental procedures, both involving functional antagonism, which could be used to reveal differences in efficacy of those β-adrenoceptor agonists which normally appear as full agonists. He suggested that the best method was that in which the β-adrenoceptor agonist, acting as the functional antagonist, caused a shift in the concentration-response lines to a muscarinic receptor agonist. Using this approach the ratio of the maximum shift produced by two β-adrenoceptor agonists is equal to the ratio of their efficacies. In the present study this method has been used in the tracheal experiments but it was not suitable for use in atrial experiments because of difficulties in obtaining concentration-response lines to carbachol on atrial rate. Therefore, with atria the alternative method was used in which the maximum responses produced by the β-adrenoceptor agonists in the presence of a muscarinic receptor agonist acting as functional antagonist, were compared. From the theoretical models proposed by Van den Brink (1973a,b) the maximum responses to β-adrenoceptor agonists in the presence of a given concentration of the muscarinic agonist will differ according to their efficacies.

These methods have been used to obtain the order of efficacies of fenoterol (Th 1165a), Me 506, Me 454 and isoprenaline. The former three drugs were selected because they were trachea-atria selective compounds and had previously been shown to give a maximum response on both trachea and atria (O'Donnell, 1972; O'Donnell & Wanstall, 1974). Also, in recent biochemical studies using adenylate cyclase assays in frog erythrocyte membranes (Mukherjee, Caron, Mullikin & Lefkowitz, 1976) Me 454 (or Cc-34) had a higher efficacy (the term intrinsic activity is used in their paper) than that of isoprenaline. In the same

**Table 1** Guinea-pig isolated tracheal preparations. Values of neg log $EC_{50}$ and % maximum contraction for carbachol in the absence and in the presence of various β-adrenoceptor agonists acting as functional antagonists.

| *β-Adrenoceptor agonist (functional antagonist)* | | | |
|---|---|---|---|
| *Compound* | *Concentration (μM)* | *neg log $EC_{50}$ carbachol* | *% maximum contraction* |
| None (control) | | 6 26 ± 0.02 (58) | 100 |
| Isoprenaline | 1 | 5.18 ± 0.07 (4) | 94 ± 3.0 |
| | 5 | 5.14 ± 0 04 (15) | 91 ± 1.8 |
| | 20 | 5.18 ± 0.04 (26) | 93 ± 1.4 |
| Fenoterol, | 10 | 5.44 ± 0.11 (7) | 98 ± 2.7 |
| | 100 | 5.26 ± 0.07 (9) | 100 ± 3.4 |
| Me 454 | 10 | 5.06 ± 0.06 (6) | 89 ± 2.3 |
| | 100 | 4.62 ± 0.05 (7)† | 85 ± 1 8 |
| Me 506 | 100 | 5.20 ± 0.03(4) | 97 ± 0.85 |
| | 1000 | 5.04 ± 0.03 (6)* | 92 ± 1.7 |

All values are mean values ± s.e. mean.

The number of different tissue preparations is given in parentheses.

† Significantly lower than value for 10 μM Me 454 (*t*-test, $P < 0.001$); * significantly lower than value for 100 μM Me 506 (*t*-test, $0.01 > P > 0.001$)

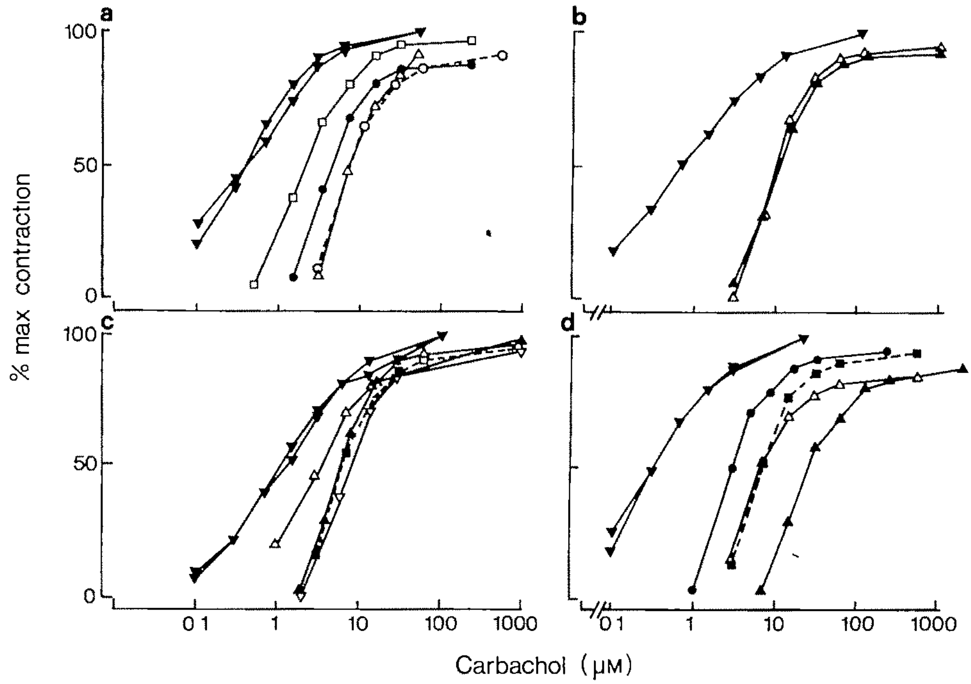

**Figure 3** Functional antagonism of carbachol by fenoterol (a and b), Me 506 (c) and Me 454 (d) on 7 preparations of isolated trachea from 4 guinea-pigs Concentration-response lines to carbachol in the absence (▼) and in the presence of various concentrations of functional antagonist are illustrated. (a) and (b) show the shift in the carbachol line by concentrations of fenoterol of 100 nM (□), 1 μM (●), 10 μM (△) and 100 μM (▲). The shift with 10 μM fenoterol is the same as that with 100 μM fenoterol and with 5 μM isoprenaline (○– – – –○). (c) shows the shift in the carbachol line by concentrations of Me 506 of 10 μM (△), 100 μM (▲) and 1 mM (▽) The shift with 1 mM Me 506 is greater than that with 20 μM isoprenaline (■– – – –■). (d) shows the shift in the carbachol line by concentrations of Me 454 of 1 μM (●), 10 μM (△) and 100 μM (▲). The shift with 100 μM Me 454 is greater than that with 20 μM isoprenaline (■– – – –■)

**Table 2** Guinea-pig isolated tracheal preparations. Values of log unit shift of carbachol concentration-response lines produced by various concentrations of β-adrenoceptor agonist together with the maximum log unit shift produced by isoprenaline on the same tissues.

| | *β-Adrenoceptor agonist* | | | |
|---|---|---|---|---|
| *Compound* | *Concentration (μM)* | *log unit shift* | *Isoprenaline max log unit shift* | *Number of different tissue preparations* |
| Fenoterol | 10 | 0.88±0.11 | 1.08±0.07 | 7 |
| | 100 | 0.99±0.05 | 1.03±0.05 | 9 |
| Me 454 | 10 | 1.24±0.04 | 1.16±0.08 | 5 |
| | 100 | 1.76±0.06*** | 1.22±0.07 | 7 |
| Me 506 | 100 | 0.88±0.08 | 0.81±0.14 | 4 |
| | 1000 | 1.11±0.06* | 0.99±0.05 | 6 |

All values are mean values ± s.e. mean.

* Significantly greater than the log unit shift with 100 μM Me 506 (Student's $t$-test) and than the max log unit shift with isoprenaline (paired $t$-test $0.05 > P > 0.01$); *** Significantly greater than the log unit shift with 10 μM Me 454 (Student's $t$-test) and than the max log unit shift with isoprenaline (paired $t$-test $P < 0.001$)

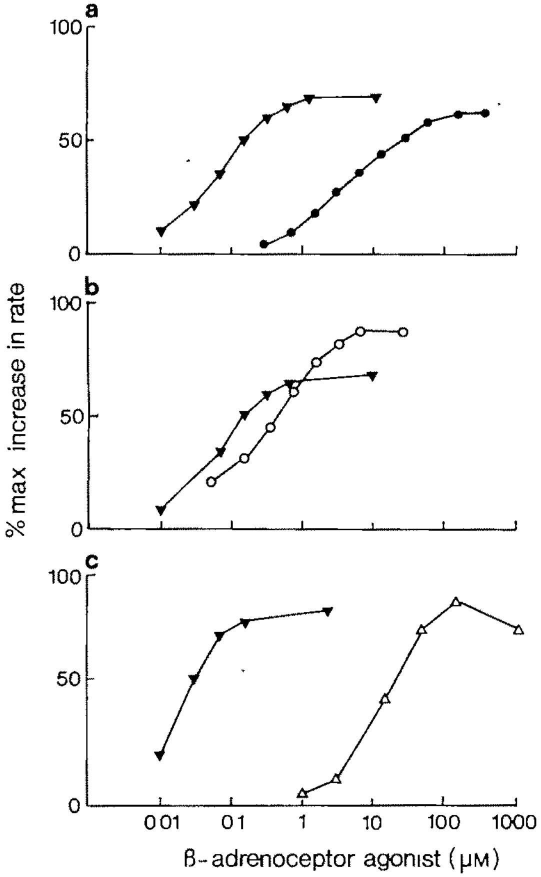

**Figure 4** Three preparations of guinea-pig isolated atria. Concentration-response lines to isoprenaline (▼), fenoterol (●), Me 454 (○), and Me 506 (△) are shown. The experiments were carried out in the presence of carbachol (300 nM in (a) and (b) and 200 nM in (c)). The figure uses one typical experiment for each β-adrenoceptor agonist to illustrate the findings that the maximum response to fenoterol was not significantly different from isoprenaline ($t=0.14$, d.f. 5, paired $t$-test); the maximum response to Me 454 was significantly greater than that to isoprenaline ($t=4.76$, d.f. 4, $0.01 > P > 0.01$ paired $t$-test) and the maximum response to Me 506 was greater than that to isoprenaline but not significantly ($t=1.14$, d f. 3, paired $t$-test).

study Cc-25 was also shown to have an efficacy greater than that of isoprenaline. A similar finding was reported by Van den Brink (1973b) in pharmacological experiments of the type described in this paper but carried out on calf trachea. Me 506 and fenoterol are resorcinolamines with the same N-alkyl substituent groups as Me 454 and Cc-25 respectively, the latter compounds being catecholamines.

In the present study the efficacies of Me 454 and Me 506 on trachea were both higher than that of isoprenaline and the efficacy of fenoterol was as high as that of isoprenaline. On the atria the efficacy of Me 454 was clearly greater than that of isoprenaline, the efficacy of Me 506 was at least equal to and possibly greater than that of isoprenaline and the efficacy of fenoterol was the same as that of isoprenaline. In other words, the three compounds examined had an efficacy at least as great as that of isoprenaline on both tissues. For Me 454, a catecholamine, it was encouraging that there was some agreement between the conclusions from the pharmacological (present study) and biochemical (Mukherjee *et al.*, 1976) approaches to efficacy (intrinsic activity) measurements. This was despite the involvement of different species and different tissues in the two experimental situations. The other two compounds examined (fenoterol and Me 506) were resorcinolamines, not catecholamines, and were not included in the study by Mukherjee *et al.* (1976). The non-catecholamines which these workers studied all had low efficacies and this led them to conclude that a catechol ring structure was necessary for full efficacy. The present pharmacological study has indicated that high efficacy is possible in the absence of a catechol structure provided that a favourable N-substituent group is present in the molecule. On the other hand, comparison of the results for Me 454 (catecholamine) and Me 506 (resorcinolamine) indicated that, if the catechol ring was also present, an even higher efficacy was feasible.

The trachea-atria selectivity of the drug soterenol may depend entirely on its low efficacy combined with a difference between the β-receptor reserves in trachea and atria since no difference was found between its affinity values on trachea and atria (Buckner & Saini, 1975). This could be the reason why soterenol was not a strikingly selective β-agonist (Buckner & Abel, 1974). Thus soterenol may not be the ideal compound to use when obtaining data from which conclusions on the similarity or difference between receptors are being made. Raper & Malta (1975) commented that the selectivity of soterenol and other similar compounds may be related more to their efficacy than to their affinity but they did not make it clear whether they were referring to low efficacy on both trachea and atria or to different efficacies on the two tissues. For other compounds which are in general more selective than soterenol, it is feasible that low efficacy and differences in tissue receptor reserves contribute to their trachea-atria selectivity but that they have, in addition, different affinities for receptors in the two tissues. For example, salbutamol is a partial agonist on guinea-pig atria (O'Donnell, 1972) and on carbachol-contracted tracheal preparations (O'Donnell & Wanstall, unpublished results) suggesting that it has a lower efficacy than isoprenaline, but there is also evidence, from $pA_2$ values, that it has a different affinity for the β-receptors in the two tissues (Raper & Malta, 1973). On the other hand the present study has shown that compounds do exist (e.g. fenoterol, Me 506 and Me 454) which are trachea-atria selective but which have efficacies at least as high as that of isoprenaline. Therefore, low efficacy cannot be invoked to explain their trachea-atria selectivity.

For those compounds without low efficacy the assumption can be made that their selectivity reflects a true difference between their affinities for the β-receptors in guinea-pig trachea and atria, although further experiments are required to confirm this. This concept is not compatible with the hypothesis that the receptor in these two tissues is of a single type (Buckner & Patil, 1971; Buckner & Abel, 1974; Buckner & Saini, 1975). The present study has also raised again the question of whether isoprenaline is really the ideal reference compound for use in pharmacological studies on receptor mechanisms in tissues containing β-receptors. We have previously illustrated how loss of isoprenaline into extraneuronal uptake sites, e.g. in the guinea-pig trachea, can influence conclusions on the selectivity of compounds (O'Donnell & Wanstall, 1976a). Now we have confirmed that isoprenaline is not the β-adrenoceptor agonist with the highest efficacy on β-adrenoceptors.

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# COMMUNICATIONS

## GABA-like properties of flurazepam and baclofen suggested by rotational behaviour following unilateral intranigral injection: a comparison with the GABA agonist muscimol

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There is much interest in the possible involvement of $\gamma$-aminobutyric acid (GABA) in mediating certain pharmacological properties of benzodiazepines (Haefely, Kulscar, Möhler, Pieri, Polc & Schaffner, 1975). Unilateral elevation of GABA levels in the zona reticulata of the rat substantia nigra (SNR) produces ipsilateral rotation under the influence of amphetamine (Dray, Fowler, Oakley, Simmonds & Tanner, 1975). A similar induction of rotational behaviour is produced by unilateral injection of the benzodiazepine chlordiazepoxide into the SNR of amphetamine-pretreated animals, suggesting an enhancement of GABA transmission by chlordiazepoxide; this effect was abolished by the GABA antagonist picrotoxin and mimicked by the GABA derivative baclofen (Waddington, 1976).

Unilateral elevation of GABA levels in SNR has recently been shown to produce contralateral rotation in the absence of treatment with dopamine agonists (Koob, Del Fiacco & Iversen, 1976); if benzodiazepines do enhance GABA transmission then their unilateral injection into SNR should similarly induce contralateral rotation without pretreatment. The effects of such injections of the potent soluble benzodiazepine flurazepam hydrochloride and baclofen were compared with those of muscimol, which has pharmacological properties suggesting GABA agonist activity (Curtis, Duggan, Felix & Johnston, 1971).

Male Sprague-Dawley rats, 150 ± 20 g, were anaesthetized with ether and given histologically confirmed unilateral stereotaxic injections of flurazepam, baclofen or muscimol into SNR in 1 µl saline; control animals received 1 µl saline. Immediately following injection animals were placed in automated rotameter bowls and rotations measured continuously for 1 hour. Control animals showed a weak ipsilateral rotation on recovery. Both flurazepam

(1–20 μg, $P<0.001$) and baclofen (5–25 ng, $P<0.001$) injections produced a vigorous dose-dependent contralateral rotation; these effects were mimicked by injections of muscimol (1 ng, $P<0.005$).

Considerable debate has centred on whether striatonigral GABA neurones are excitatory or inhibitory on nigrostriatal dopamine neurones (Dray & Straughan, 1976) whose asymmetric activity is presumed to underlay rotational behaviour; this demonstrated induction of contralateral rotation in the absence of any pretreatment suggests an excitatory process. Whether or not this is the case flurazepam produces effects identical to those produced by unilateral elevation of SNR GABA levels and SNR injections of both the GABA analogue baclofen, recently shown electrophysiologically to produce GABA-like depression of activity that is antagonized by the GABA antagonist bicuculline (Puil, Krnjevic and Werman, 1976), and the GABA agonist muscimol. These results suggest that baclofen may have some GABA agonist activity and further emphasize the GABA-like properties of benzodiazepines; whether this action is related to their clinical anxiolytic effect remains to be determined.

JLW is an MRC Scholar. Flurazepam, baclofen and muscimol were gifts from Roche Products Limited, Ciba Laboratories and Royal Dutch Shell respectively.

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# Inhibition of dopaminergic activity in the extrapyramidal and limbic systems by γ-acetylenic GABA

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There is considerable evidence that γ-aminobutyric acid (GABA) acts as an inhibitory transmitter in the nigrostriatal pathway by inhibiting the ascending dopaminergic pathway at the level of the substantia nigra (Dray & Straughan, 1976). There is less evidence for a similar inhibitory role of GABA in the limbic system.

Using a new irreversible catalytic inhibitor of GABA-transaminase, γ-acetylenic GABA (GAG; RMI 71645; Jung, Lippert, Metcalf, Schechter, Böhlen & Sjoerdsma, 1977), we have examined inhibition of both the extrapyramidal and limbic dopaminergic pathways.

Three techniques were used to estimate catecholamine turnover in selected rat brain areas 4 h following GAG (100 mg/kg i.p.) administration: (a) α-methyl-*p*-tyrosine (AMPT)-induced disappearance of dopamine and noradrenaline, (b) homovanillic acid (HVA) concentrations, (c) [$^3$H]-dopamine formation following [$^3$H]-L-DOPA treatment. GAG decreased AMPT-induced dopamine disappearance in the striatum and olfactory tubercles ($P<0.005$). [$^3$H]-dopamine formation was also decreased in these regions (30 and 26% respectively, $P<0.05$) as was HVA concentration (61 and 49%, $P<0.005$). On the other hand, in the hypothalamus a small but significant ($P<0.02$) increase in dopamine turnover was found by the AMPT method and hypothalamic HVA was also increased (141%, $P<0.02$). Noradrenaline turnover was decreased in the olfactory tubercle and unchanged in the hypothalamus.

In a further series of experiments, rats received unilateral injections into the substantia nigra of 20 or 40 μg GAG in 2 μl saline or saline alone. Five hours later the rats were injected i.p. with either amphetamine (3–5 mg/kg) or apomorphine (2–6 mg/kg) and the number of rotations per minute were recorded for the following 2 hours. GAG-treated animals showed a consistent dose and time related ipsilateral turning with both amphetamine and apomorphine (e.g. 6.1 ± 0.7 turns/min after 6 mg/kg apomorphine; mean ± s.e., $n=8$), whereas saline injected rats did not turn. This effect is consistent with inhibition of the nigrostriatal dopaminergic pathway by $\gamma$-acetylenic GABA.

For investigations of the limbic system, rats were injected bilaterally with GAG into the nucleus accumbens (10 μg/side in 2 μl) 3 h before the bilateral injection of dopamine (25 μg in 2 μl/side). All rats were dosed orally with isocarboxazid (100 mg/kg) 2 h before receiving dopamine. Motor activity was measured in an activity meter for 3 h following dopamine injection. GAG significantly reduced the dopamine-activated increase in motor activity, consistent with a GABA-mediated inhibition of the nucleus accumbens.

To verify the specificity of the effect of $\gamma$-acetylenic GABA on the two dopaminergic pathways a number of injections were made in various regions close to the substantia nigra and the nucleus accumbens. Injections more than 1 mm distal from the required area (verified histologically) were without effect on either turning or motor activity.

GABA-transaminase activity was estimated in samples obtained by micropuncture from regions in and close to the substantia nigra or nucleus accumbens. Injections of GAG into the substantia nigra and nucleus accumbens produced 88–90% inhibition in these areas. Fifty per cent or greater inhibition was found in areas approximately 1 mm from the site of injection suggesting a limited diffusion of $\gamma$-acetylenic GABA.

In conclusion the results suggest an inhibitory role for GABA in both the extrapyramidal and limbic systems.

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# Rotational responses to the putative serotonin agonist 5MeODMT following unilateral 5,6-DHT lesions of the median forebrain bundle: a possible role for 5-HT in the control of rotational behaviour

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Asymmetric lesions of the medial raphé nucleus, which produce unilateral depletion of forebrain serotonin (5-HT), produce contralateral rotation in rats when treated with dopamine agonists (Costall, Naylor, Marsden & Pycock, 1976). Together with evidence indicating a serotonergic modulation of rotational responses to dopamine agonists in rats with unilateral 6-hydroxydopamine (6-OHDA) lesions of ascending dopamine (DA) pathways (Milson & Pycock, 1976; Waddington, 1977), this suggests the possibility of inducing a rotational response to 5-HT agonists in rats with unilateral lesions of ascending 5-HT pathways. This was investigated using unilateral lesions of the median forebrain bundle (MFB) induced with the indolamine neurotoxin 5,6-dihydroxytryptamine (5,6-DHT) in a comparison with lesions induced with 6-OHDA. For both types of lesions, rotational responses to the DA agonist apomorphine were compared with those following treatment with the putative 5-HT agonist 5-Methoxy-*N*,*N*-dimethyltryptamine (5MeODMT).

Male Sprague-Dawley rats, 150 ± 20 g, received unilateral stereotaxic injections of 6-OHDA (8 μg/4 μl saline; $n = 14$) or 5,6-DHT (5 μg/4 μl saline; $n = 14$) into the MFB. Eight days post lesion all animals received i.p. injections of apormorphine (1 mg/kg); on day 11 animals received 5MeODMT (2 mg/kg), 45 min after pretreatment with the monoamine oxidase inhibitor nialamide (75 mg/kg i.p.). Rotational behaviour was recorded in automated rotameter bowls for continuous recording of all rotations and these were assessed both with conventional statistical techniques and following orthogonal polynomial transformation. Rats were sacrificed 14 days post lesion and striata assayed spectrophotofluorimetrically for DA and 5-HT content.

6-OHDA and 5,6-DHT lesions produced 72.3% ($P < 0.001$) and 83.9% ($P < 0.001$) depletions of striatal DA respectively; there was no significant difference between DA depletions following the two lesion procedures ($P < 0.05$). 5-HT levels were reduced by 12.5% ($0.05 < P < 0.1$) and 52.5% ($P < 0.001$) following 6-OHDA and 5,6-DHT respectively; 5-HT depletion with 5,6-DHT exceeded that with 6-OHDA ($P < 0.001$).

6-OHDA animals showed the expected contralateral rotation to apomorphine; 5,6-DHT animals showed a faster onset and offset of apomorphine-induced contralateral rotation ($P < 0.05$) as previously described (Waddington, 1977), though mean rotations were unaltered. 5MeODMT induced a mild ipsilateral rotation in 6-OHDA-lesioned animals treated with nialamide; however, this response was more vigorous in 5,6-DHT-lesioned animals ($P < 0.05$). Nialamide alone failed to induce rotation in animals from either group.

The equivalence of DA depletions produced by either lesion procedure suggests that these results may be dependent upon the differing degrees of 5-HT depletion they produce. We are led to suggest that 5MeODMT-induced rotation may be due to a direct activation of forebrain 5-HT receptors on the lesioned side rendered supersensitive by denervation induced by 5,6-DHT and, to a much lesser extent, by a non-specific effect of 6-OHDA; these 5-HT receptors may be in opposition to striatal DA receptors with regard to determining the direction of movement. The relative roles of receptors in the striatum or limbic forebrain areas in mediating these effects, however, remain to be determined.

J.L.W. is an MRC Student.

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# 5-HT antagonists inhibit neuroleptic and morphine antagonism of the hyperactivity induced by DA from the nucleus accumbens

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The injection of dopamine (DA) into the nucleus accumbens (ACB) of rats induces marked hyperactivity which is specifically antagonized by neuroleptic agents administered either peripherally or directly into the ACB (Pijnenburg, Honig & Van Rossum, 1975; Costall, Naylor & Pinder, 1976). This antagonism of the DA effect, which is also achieved using morphine (Fortune, unpublished data), has been interpreted almost exclusively in terms of DA receptor blockade (Costall & Naylor, 1976). However, injections of 5-hydroxytryptamine (5-HT) into the ACB also antagonizes the DA-induced hyperactivity (Costall, Naylor, Marsden & Pycock, 1976) and preliminary evidence indicates that the more enhanced effects of neuroleptic agents and morphine on motor function, namely catalepsy induction, involves an enhanced 5-HT action (Kostowski, Gumulka & Czlonkowski, 1972; Costall, Fortune, Naylor, Marsden & Pycock, 1975; Costall, Fortune & Naylor, 1977). In the present study we investigate the possibility that neuroleptic agents and morphine may antagonize the hyperactivity induced by DA from the ACB via a similar mechanism.

Animals were prepared for intracerebral injection into the ACB using the techniques of Costall & Naylor (1976) and drugs were injected in a volume of 1 µl. DA (5–50 µg) administered bilaterally into the ACB of chronically implanted rats pretreated with nialamide (100 mg/kg i.p., 2 h) induced a dose-dependent hyperactivity: this was recorded by placing rats in individual perspex cages fitted with photocells and measuring the number of interruptions of the light beam. The hyperactivity induced by 50 µg DA was antagonized by subsequent bilateral injections of 5-HT (6.3–25 µg), fluphenazine (6.3–25 µg) and morphine (0.5–5 µg) into the ACB. The antagonism of DA-induced hyperactivity by submaximal doses of 5-HT (12.5 µg), fluphenazine (12.5 µg) and morphine (1 µg) was significantly reduced or abolished by the administration of methysergide (0.063–1 mg/kg i.p.) or cyproheptadine (1–25 mg/kg i.p.). Both methysergide (1 mg/kg i.p.) and cyproheptadine (2.5 mg/kg i.p.) failed to modify the hyperactivity induced by 5 or 50 µg DA injected into the ACB.

It is concluded that the ability of morphine or of a neuroleptic agent such as fluphenazine to antagonize the hyperactivity induced by DA from the ACB may be associated with an enhancement of 5-HT function either concomitant or subsequent to a DA receptor blockade. The possibility that the locus of the 5-HT action may be within the mesolimbic circuits, suggested as possible substrates for the clinical antipsychotic action of the neuropleptic agents, may be relevant to an understanding of the action of an antipsychotic agent such as clozapine which, although having only weak blocking action on the DA receptor, is able to enhance 5-HT function (Ruch, Asper & Bürki, 1976).

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## Possible importance of 5-hydroxytryptamine in neuroleptic-induced catalepsy in rats

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The state of catalepsy in rodents following systemic administration of neuroleptic agents has been closely associated with blockade of central dopamine (DA) receptors (Fog, 1972): the intensity of catalepsy presumably relating to the degree of inhibition of dopamine function. Conversely, stimulation of DA receptors by classical DA agonists such as apomorphine and amphetamine elicits hyperactivity and stereotypy. However the role of other neurotransmitters known to influence DA mechanisms are rarely considered in these behavioural states. In particular 5-hydroxytryptamine (5-HT) manipulation is known to modify DA-dependent behaviours, and blockade of central 5-HT mechanisms has been reported as reducing neuroleptic-induced catalepsy in rats (Costall, Fortune, Naylor, Marsden & Pycock, 1975; Maj, Mogilnicka & Przewlocka, 1975).

We have further investigated the effect of 'classical DA agonists' and a number of drugs known to modify central 5-HT mechanisms on catalepsy induced by neuroleptic drugs in rats. Rather contrary to present simplified concepts, we have shown that proposed DA agonists can in fact potentiate neuroleptic-induced catalepsy. Amphetamine (4 mg/kg i.p.) administered in combination with a variety of neuroleptic drugs (haloperidol, 1 mg/kg i.p., fluphenazine, 1 mg/kg i.p.; pimozide, 5 mg/kg i.p.) significantly enhanced the cataleptic response. Lower doses of amphetamine (1 and 2 mg/kg) had no significant effect on the neuroleptic-induced catalepsy, while a high dose (8 mg/kg) tended to reverse this behaviour and induce stereotypy. Similarly apomorphine (0.12–0.5 mg/kg s.c.) was shown to potentiate haloperidol (1 mg/kg) catalepsy significantly. Higher doses of apomorphine tended to reverse the cataleptic state. L-DOPA (80 and 160 mg/kg i.p.), when administered in combination with the dopamine uptake blocking agent nomifensine (10 mg/kg i.p.), also caused significant potentiation of the cataleptic response to haloperidol (1 mg/kg).

The proposed 5-HT agonists quipazine (5–40 mg/kg i.p.) and 5-methoxy-*N*,*N*′-dimethyltryptamine (1–4 mg/kg i.p.) and the 5-HT precursor 5-hydroxytryptophan (10–200 mg/kg i.p.) potentiated the cataleptic response of the neuroleptic haloperidol (1 mg/kg) as did the selective 5-HT uptake blocking compounds ORG 6582 (1.25–10 mg/kg i.p.) and FG 4963 (femoxetine, 5–40 mg/kg i.p.). Conversely, treatment with the 5-HT antagonists cyproheptadine

(0.06–0.25 mg/kg i.p.) or methysergide (10–40 mg/kg i.p.) decreased the cataleptic effect of haloperidol (4 mg/kg).

The results suggest that 5-HT function can modify the state of catalepsy relating to blockade of central DA mechanisms. Thus, increasing the level of 5-HT neurotransmission apparently enhances catalepsy, but only after blockade of DA receptors has been established.

The rather surprising result that potential DA agonists can, at certain doses, potentiate neuroleptic-induced catalepsy may be explained in terms of possible 5-HT mechanisms. It is known for example that amphetamine can release 5-HT (Fuxe & Ungerstedt, 1970), apomorphine increase 5-HT turnover (Grabowska, 1975), and that L-DOPA can displace 5-HT from central serotonergic nerve endings (Algeri & Cerletti, 1974).

In view of these findings one should perhaps be wary of interpreting various behavioural responses in rodents seen after the 'classical' DA agonists purely in terms of DA mechanisms.

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# Interactions of substituted benzamide drugs with cerebral dopamine pathways

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Substituted benzamide drugs exhibit behavioural and biochemical properties consistent with a blockade of cerebral post-synaptic dopamine receptors (Peringer, Jenner, Donaldson, Marsden & Miller, 1976; Elliott, Jenner, Huizing, Marsden & Miller, 1976), thereby resembling classical neuroleptic compounds. However, in contrast to classical neuroleptics, substituted benzamides do not consistently inhibit the dopamine stimulated-adenylate cyclase system from rat striatum. We have examined other biochemical interactions of three substituted benzamide drugs, namely, metoclopramide (*N*-[diethylaminoethyl]-2-methoxy-4-amino-5-chlorobenzamide), sulpiride (*N*-[1′-ethyl-2′-pyrrolidinylmethyl]-2-methoxysulphamoylbenzamide) and clebopride (*N*-[*N*′-benzylpiperidin-4′yl]-4-amino-5-chloro-2-methoxybenzamide) with cerebral dopamine systems *in vivo* and *in vitro*.

Metoclopramide (100 mg/kg i.p.), sulpiride (100 mg/kg i.p.), clebopride (10 mg/kg i.p.) or haloperidol (0.1 mg/kg i.p.) elevated striatal and mesolimbic HVA and DOPAC in rats ($P < 0.001$). Pretreatment with gammahydroxybutyrolactone (GOBA; 750 mg/kg), an inhibitor of impulse flow, prevented the rise in the level of dopamine metabolites caused by all of these drugs.

Metoclopramide (20 mg/kg i.p.), sulpiride (50 mg/kg i.p.), clebopride (10 mg/kg i.p.) and haloperidol (0.1 mg/kg i.p.) raised striatal and mesolimbic HVA and DOPAC levels in the mouse ($P < 0.02$). The HVA elevation caused by haloperidol was partially blocked by atropine (50 mg/kg i.p.) pretreatment ($P < 0.005$); DOPAC levels were unaffected ($P > 0.05$). Metoclopramide and clebopride-induced increases in HVA were not affected by atropine pretreatment ($P > 0.05$) but the rise induced by sulpiride was reduced by atropine ($P < 0.02$). DOPAC levels were unaffected by atropine following clebopride and sulpiride administration but mesolimbic DOPAC in metoclopramide treated animals was reduced ($P < 0.01$).

Incorporation of metoclopramide, sulpiride and clebopride ($10^{-10}$ to $10^{-6}$ M) into the [$^3$H]haloperidol ($2 \times 10^{-9}$ M) labelled dopamine receptor binding model from rat striatum (Creese, Burt & Snyder, 1976) caused displacement of haloperidol with $IC_{50}$ values of $4.2 \times 10^{-7}$ M, $3.6 \times 10^{-7}$ M and $2.0 \times 10^{-8}$ M respectively. These compare with values of $2.4 \times 10^{-9}$ M and $7.9 \times 10^{-9}$ M obtained for haloperidol and (+)-butaclamol respectively.

Uptake and release of [$^3$H]-dopamine by striatal synaptosomal particles was weakly affected by metoclopramide ($IC_{50}$ $5 \times 10^{-4}$ M and $EC_{50}$ $2 \times 10^{-5}$ M respectively), but was unaffected by sulpiride. Clebopride did not inhibit dopamine uptake, but had a marked effect on release ($EC_{50}$ $1 \times 10^{-7}$ M). These values compare with an uptake $IC_{50}$ value of $9 \times 10^{-9}$M for nomifensine and a release $EC_{50}$ value of $1 \times 10^{-8}$ for amphetamine.

These data suggest that the substituted benzamides investigated do interact *in vitro* with dopamine receptors, although they have no consistent effect on dopamine-sensitive adenylate cyclase, nor do they consistently influence presynaptic dopamine uptake or release. They do cause an increase in cerebral dopamine metabolites, which is dependent on nerve impulse flow as judged by the effects of GOBA. Such an increase in HVA is sensitive to atropine in the case of sulpiride, but not in the case of metoclopramide and clebopride. Thus, substituted benzamides resemble classical neuroleptics in some respects, but differ in others.

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## Antipodal central effects of dopamine and apomorphine

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Cohen & Berkowitz (1975) suggested there were two types of dopamine receptor, based on the response of rat aortic strips to dopamine and to apomorphine. Cools & van Rossum (1976) postulated that there were 'two types of dopamine site' in the central nervous system. Clear-cut differences (Table 1) in the effects of dopamine and apomorphine infused into the hypothalamus and the third cerebral ventricle were observed in young and adult chickens (*Gallus domesticus*).

Thus dopamine (0.1 µmol) infused into the hypothalamus of young chicks pretreated with mebanazine (10 µmol/100 g i.v. 18 h and 1 h previously) induced behavioural and electrocortical sleep, suppressed vocalization, body temperature declining up to 6°C at thermoneutrality and mean $CO_2$ elimination falling 55%, effects lasting 4–6 hours. In contrast, apomorphine (0.05 µmol) infused into the identical site evoked behavioural and electrocortical arousal, vocalization and pecking, accompanied by an 0.5°–1°C increase in body temperature at thermoneutrality, and a mean increase in $CO_2$ elimination of 70%. The effects on body temperature lasted about 40 min, and 20–30 min for other variables. Apomorphine (0.025 µmol/100 g i.v.) evoked behavioural and electrocortical arousal in chicken encéphale isolé preparations. Similar effects to the doses infused into the hypothalamus were elicited by dopamine (0.5 µmol) and apomorphine (0.25 µmol) given into the third cerebral ventricle of adult fowls at thermoneutrality.

**Table 1** Effects of dopamine and apomorphine infused into the hypothalamus and third cerebral ventricle of chickens

| | *Dopamine* | *Apomorphine* |
|---|---|---|
| Behaviour | Sleep | Arousal |
| Electrocortical activity | Slow wave, large amplitude | Fast frequency, small amplitude |
| Posture | Standing or squatting, wings lowered | Standing (wing abduction in adult only) |
| Vocalization | Decreased | Increased |
| Body temperature | Decreased | Increased |
| $CO_2$ elimination | Decreased | Increased |
| Effective antagonists | Phenoxybenzamine | Spiroperidol (encéphale isolé) |
| Ineffective antagonists | Spiroperidol, propranolol | Phenoxybenzamine, propranolol |
| Effects of amphetamines | Dissimilar to those of dopamine | Resembled those of apomorphine |

The effects of dopamine but not apomorphine were attenuated by phenoxybenzamine (0.1 µmol) given previously into the hypothalamus. In contrast, the effects of apomorphine were attenuated by spiroperidol (0.0025 µmol/100 g i.v.). Phentolamine, even in doses as small as 0.005 µmol given centrally, elevated body temperature 1°C for 4–5 h, so precluding testing with an adequate dose-ratio against the agonists. Propranolol was ineffective against both substances. Whereas the effects of apomorphine resembled those of dexamphetamine, doses of methysergide (0.01 µmol/100 g i.v.) that prevented arousal with dexamphetamine did not affect response to apomorphine. The results are compatible with the existence of two types of central dopamine receptor in chickens.

B.A.K. is an MRC Student.

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## Origin of dopaminergic afferents to the rat frontal cortex

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(Introduced by L.L. IVERSEN)

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Dopamine is present in two areas of the rat frontal cortex, the medial prefrontal and cingulate cortex, where it is believed to be contained in nerve fibres (Thierry, Stinus, Blanc & Glowinski, 1973; Lindvall, Bjorklund, Moore & Stenevi, 1974). Using a sensitive radioenzymatic assay for dopamine (DA) and noradrenaline (NA) (Cuello, Hiley & Iversen, 1973) we have mapped the distribution of these two catecholamines in the areas and layers of the prefrontal and cingulate cortex. In particular, we investigated the response of these two catecholamines to lesions designed to deplete selectively either DA or NA in the frontal cortex.

The quantities of DA and NA in the dorsal, and medial prefrontal and cingulate cortical areas are indicated in Table 1. The unilateral injection of 6-hydroxydopamine (6OHDA) 8 µg in 2 µl saline (ascorbic acid 1 mg/ml) into the ascending NA projections in the central tegmental tract (Mason & Iversen, 1975) resulted in an almost complete depletion of the frontal cortex NA. In contrast the content of DA was increased in those areas of frontal cortex believed to contain DA terminals and reduced in areas which contain mainly NA. Thus in the dorsal prefrontal cortex area DA is probably a precursor of

**Table 1** The effects of various lesions on the catecholamine content of the rat frontal cortex

| | Cortical area assayed | Dopamine content ng/g | % change relative to control | Noradrenaline content ng/g | % change relative to control |
|---|---|---|---|---|---|
| Control samples | +DPFC | 24.6 ± 3.7 (6) | — | 134 ± 21 (6) | — |
| | +MPFC | 74.5 ± 11.6 (6) | — | 137 ± 23 (6) | — |
| | +CgFC | 48.0 ± 6.4 (5) | — | 114 ± 29 (6) | — |
| 6-hydroxydopamine (8 µg/2 µl saline) into ascending noradrenaline bundle | DPFC | 14.5 ± 5.6 (7) | −41% | 3.2 ± 3.3* (7) | −97.2% |
| | MPFC | 104 ± 25* (7) | +39% | 8.2 ± 3.6* (7) | −95.4% |
| | CgFC | 72.4 ± 22.3* (7) | +50% | 14.8 ± 7.1* (7) | −87.0% |
| Electrolytic lesion in ventral tegmental area | DPFC | 20.3 ± 14.4 (6) | −6.7% | 146 ± 7 (6) | +8.9% |
| | MPFC | 24.8 ± 13.4* (6) | −63% | 136 ± 19 (6) | +0.7% |
| | CgFC | 32.2 ± 3.9* (6) | −33% | 125 ± 14 (6) | +9.6% |
| 6-hydroxydopamine (4 µg/µl saline) into lateral substantia nigra | DPFC | 23.1 ± 3.9 (6) | −7% | 138 ± 21 (6) | +2.1% |
| | MPFC | 76.3 ± 41.4 (6) | +3% | 120 ± 19 (6) | −13.5% |
| | CgFC | 36.3 ± 2.5* (6) | −25% | 109 ± 22 (6) | −4.4% |

+ DPFC—Dorsal prefrontal cortex, MPFC—Medial prefrontal cortex, CgFC—Cingulate cortex. Numbers of determinations given in brackets. Statistical analysis by paired *t*-test. Significance (*) taken at $P<0.05$.

NA. Electrolytic lesions in the ventral tegmental area resulted in a significant depletion of DA from both the medial prefrontal and cingulate cortex but produced no change in cortical NA. The depletion of DA from the cingulate cortex was not complete and indicated the possible presence of a separate group of DA terminals whose cell bodies of origin were outside the ventral tegmentum. Unilateral injections of 6OHDA (4 μg/1 μl) into the substantia nigra produced a small but significant depletion of DA from the superficial layers of the cingulate cortex. These results are consistent with previous fluorescent histochemical findings (Lindvall *et al.*, 1974).

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## The actions of cholinomimetics and catecholamines on rat substantia nigra neurones

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Acetylcholine and choline acetylase occur in the substantia nigra (Cheney, Le Fevre & Racagni, 1975) and acetylcholine excites nigra cells (Crossman, Walker & Woodruff, 1974a; Dray & Straughan, 1976). Recent evidence suggests that dopamine is released from dopamine-containing zona compacta neurone dendrites (Geffen, Jessell, Cuello & Iversen, 1976) and iontophoretically applied dopamine produces inhibition or excitation (Dray, Gonye, Oakley & Tanner, 1976). Although there is little noradrenaline in the substantia nigra, histochemical studies reveal a small population of noradrenaline-containing terminals in the zona reticulata. In this study we have examined the characteristics of the acetylcholine and catecholamine receptors of nigra neurones.

Experiments were performed on 150 g female Wistar rats, anaesthetized with urethane (1.5–2 g/kg i.p.). Extracellular recordings were made from single substantia nigra neurones using multibarrel glass microelectrodes (Crossman *et al.*, 1974b). Drugs were applied iontophoretically. The iontophoretic barrels contained acetylcholine, nicotine, carbachol, furtrethonium, atropine, physostigmine, dopamine, adrenaline and noradrenaline, all 0.2 M, pH 4–5.

Acetylcholine has predominantly excitatory effects applied iontophoretically to nigra cells. Of 190 cells tested, acetylcholine (20–60 nA) excited 105 and inhibited 20. Nicotine (30–60 nA) excited 41 and inhibited 2 of 70 cells tested. Furtrethonium (20–60 nA) excited 11 and inhibited 3 of 19 cells tested. Carbachol (20–60 nA) excited 15 and inhibited one of 19 cells tested. All four carbachol responses tested with atropine (40 nA for 45 s) were reversibly inhibited. Atropine blocked two of three actylcholine excitations and both nicotine excitations on which it was tested. Physostigmine (50 nA) applied prior and concurrently with acetylcholine potentiated the acetylcholine response on all five occasions.

Close correlation occurred between the effects following application of noradrenaline and adrenaline to nigra cells. Of 14 cells tested both adrenaline (30–60 nA) and noradrenaline (30–60 nA) excited three cells, inhibited five cells and three gave biphasic responses to both compounds; three cells were unaffected by adrenaline and two of these were similarly unaffected by noradrenaline while the third gave a biphasic response. No such correlation occurs between dopamine and noradrenaline responses. Of 13 cells excited by dopamine (30–60 nA), noradrenaline (30–60 nA) excited four, inhibited three, one was biphasic and five were unaffected. Of eight cells inhibited by dopamine, noradrenaline inhibited five, excited one and had no effect on two. Of 12 cells unaffected by dopamine, noradrenaline inhibited three, excited two, two were biphasic and five were unaffected.

It is suggested that cholinergic excitations are produced by stimulation of a mixed nicotinic/muscarinic receptor while inhibitions may be muscarinic. The catecholamine results are consistent with two populations of adrenergic receptors, one stimulated by dopamine and one by noradrenaline and adrenaline.

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## Effects of C-fragment on brain stem neurones in the cat

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The C-fragment of β-lipotropin is a potent analgesic when injected into the cerebral ventricles of the cat (Feldberg & Smythe, 1976, 1977).

We have compared the actions of C-fragment with those of morphine and methionine-enkephalin by iontophoretic application from micropipettes to neurones in the brain stem of decerebrate cats. The neurones tested were in the periaqueductal gray and other medial areas of the mid-brain, in nucleus raphe magnus, and in the medial bulbar reticular formation. Most cells were strongly inhibited by C-fragment while the remainder (6 out of 82) were unaffected, and the time course of the inhibition was similar to that of the iontophoretic application. Morphine and met-enkephalin had depressant effects on the same neurones, except that morphine produced a biphasic response (inhibition followed by excitation) in two neurones in the mid-brain on which C-fragment exerted only a depressant action. Naloxone (Endo Laboratories) applied by iontophoresis did not block the effects of C-fragment, morphine or met-enkephalin in the areas tested and occasionally exhibited agonist activity.

Although it is difficult to compare potencies with the iontophoretic method, the results suggest that C-fragment is more potent than met-enkephalin since comparable effects were obtained with smaller currents. As C-fragment has more charged side groups than met-enkephalin fewer ions will be ejected by the same current, and it is also likely to be less mobile than the smaller peptide. These considerations suggest that C-fragment is more potent than met-enkephalin in depressing the activity of neurones in the brain stem.

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## Glutamic acid: High-affinity binding to cerebellar membranes

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Although the cerebellum is low in all known neurotransmitters, glutamate is present in abundance (Johnson & Aprison, 1971), and recent neurochemical evidence (Hudson, Valcana, Bean & Timiras, 1976; Young, Oster-Granite, Herndon & Snyder, 1974) suggests that this amino acid may be the natural transmitter of the interneuronal granule cells which provide the main excitatory input to the Purkinje cells.

We have investigated the binding of highly labelled L-[$^3$H]-glutamate (34 Ci/mmol) to rat cerebellar membranes. Specific binding was determined by subtraction of the non-specific binding component, which persisted in the presence of a 10,000 fold excess of unlabelled glutamate. The time course of specific binding was relatively slow, with equilibrium being attained after approximately 10 min, whilst non-specific binding was essentially instantaneous. Specific binding, unlike the non-specific component, was saturable and exhibited at least two components. The high-affinity system only has been investigated and was found to possess an apparent $K_D = 1.27$ µM and a binding capacity of 31.8 nmol/mg protein. Specific binding was pH and temperature sensitive and was optimal under physiological conditions. Freezing of the membranes led to a rapid and progressive loss of all specific binding properties. The specific binding was found to be associated primarily with neuronal membranes, since preparations from lung, plasma, striated muscle and kidney exhibited minimal specific binding. It is suggested that high-affinity binding of glutamate to cerebellar membranes may represent combination with its physiological receptor.

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## Effects of central depressant drugs on the isolated hemisected spinal cord of the immature rat

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The immature rat spinal cord (Konishi & Otsuka, 1974) provides a convenient preparation *in vitro* for the investigation of drug receptors of the mammalian central nervous system. In 31 preparations responses of motoneurones and in some cases primary afferent terminals were elicited with both applied L-glutamate, L-homocysteate, substance P or γ-aminobutyric acid (GABA) and the effect of a series of depressant drugs on these responses was observed. Procaine hydrochloride (1 mM) was included in the perfusion medium at all times to block indirect activity (Evans & Watkins, 1975).

The psychotropic drugs chlorpromazine (50 µM), haloperidol (50 µM) and diazepam (50 µM) potentiated responses of motoneurones to L-homocysteate and to a lesser extent L-glutamate. Diazepam depressed GABA-induced primary afferent depolarization and haloperidol markedly enhanced GABA-induced primary afferent depolarizations.

The less specific depressants meprobamate (1 mM), mephenesin (1 mM), pheneturide (0.5 mM) and pentobarbitone (0.2 mM) produced depression of all motoneurone responses.

It seems possible that the potentiation of responses observed with chlorpromazine and haloperidol may relate to their reported inhibitory actions on amino acid uptake systems (Iversen & Johnston, 1971; Balcar & Johnston, 1972).

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# The effect of 40 mM potassium and electrical stimulation on the efflux of [$^3$H]-GABA from rat dorsal medulla *in vivo* and *in vitro*

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There have been two independent reports that 40 mM potassium causes an increase in the efflux of γ-aminobutyric acid (GABA) from the rat cuneate nucleus *in vivo* (Roberts, 1974; Assumpcao, Bernardi, Dacke & Davidson, 1977). We have also investigated the efflux of [$^3$H]-GABA from that part of the dorsal medulla containing the cuneate nucleus and our experiments do not support the conclusions of these previous workers.

Rats were anaesthetized with urethane (1.25 g/kg), the dorsal surface of the medulla was exposed and an incubation cup formed by placing a small length of tubing (3 mm internal diameter) on the pial surface and sealing it in place with silicone grease. Twenty µl of a Krebs solution containing $6.6 \times 10^{-6}$ M [$^3$H]-GABA and $1.2 \times 10^{-4}$ M [$^{14}$C]-sucrose as a spacemarker ($2.8 \times 10^6$ dpm each) was placed in the cup for 30–60 min. Normal Krebs solution was then superfused over the surface at a rate of 1 ml per 10 min for 30–120 min after which a change was made to an isotonic solution containing 40 mM potassium. The radioactivity in each 10 min collection was counted after adding 5 ml Instagel (Packard) and a few drops of formic acid and quenching was estimated from the external standard channels ratio. Although multiphasic $^3$H efflux curves were observed with small deflections immediately following the change-over to high potassium this only occurred in 5 out of 15 experiments and was always accompanied by a corresponding increase in $^{14}$C efflux.

The efflux of [$^3$H]-GABA from 0.4 mm slices of rat dorsal medulla was studied *in vitro* as described for rat cerebral cortex slices by Srinivasan, Neal & Mitchell (1969). The radioactivity in each 2 min collection (1–1.5 ml) was estimated as before and the efflux was followed for 40 min before changing to an isotonic solution containing 40 mM potassium. In all 8 experiments no deflections were observed. In contrast, 40 mM potassium caused a large increase in efflux of $^3$H from rat cortical slices similar to that described by Srinivasan *et al.* (1969). In 6 experiments with slices from rabbit dorsal medulla 40 mM potassium again failed to alter $^3$H washout. The efflux of $^3$H from medulla slices could be greatly increased by electrical stimulation (rectangular, 5 msec, 20 mA pulses; 60/sec for 30 s in every 2 min). This stimulation did not alter the efflux of $^{14}$C and the effect on $^3$H could be prevented by previous exposure to high potassium.

In view of these results it is suggested that any small changes in the efflux of $^3$H from the rat cuneate nucleus *in vivo* corresponding to the superfusion of high potassium solutions may be artifactual, for instance due to shrinkage of the extracellular space (Bourke & Tower, 1966; Roberts, 1976). However the specific electrically evoked release of [$^3$H]-GABA from slices of rat and rabbit dorsal medulla lends support to the hypothesis that GABA may be a transmitter at this site.

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# t-Butyl bicyclo phosphate: a convulsant and GABA antagonist more potent than bicuculline

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Bicuculline is now widely accepted as a selective GABA antagonist (Curtis & Johnston, 1974) and is at present the most potent on mammalian systems. We previously studied a series of convulsant compounds of the formula 4(R)-1-phospha 2,6,7 trioxabicyclo (2,2,2) octane-1-oxide (R-PTBO where R = alkyl group) and showed that the isopropyl derivative (IPTBO) was equipotent with bicuculline as a GABA antagonist (Bowery, Collins & Hill, 1967a; Bowery, Collins, Hill & Pearson, 1976b). Other alkyl substituted PTBO derivatives have now been studied, n-propyl (n-ProPTBO), n-butyl (nBPTBO), s-butyl (s-BPTBO), t-butyl (t-BPTBO), methyl (MPTBO) and pentyl (PPTBO), and our results indicate that the t-BPTBO derivative is more potent than bicuculline.

Convulsant potency was determined by intravenous injection in adult mice. GABA antagonism was assessed from the depression of depolarizing responses to GABA in the frog spinal cord and rat superior cervical ganglion as described previously (Bowery *et al.*, 1976b). Relative potencies were obtained by comparison with IPTBO and bicuculline at concentrations required to inhibit responses to fixed submaximal doses of GABA by 50%. This method was adopted since previous experiments have indicated that the PTBO derivatives appear to act non-competitively (Bowery *et al.*, 1976a).

All the derivatives had similar actions to those already described for IPTBO (Bowery *et al.*, 1976a, 1976b). Convulsions consisted of rapid clonic jerks leading to tonic extension with larger doses. $CD_{100}$ values are shown in Table 1. Responses to GABA (0.5–4 mM) in the frog spinal cord were readily antagonized by the PTBO derivatives whereas response to glycine and glutamate were unaffected. The derivatives also antagonized the depolarizing action of GABA (1–300 µM) in the superior cervical ganglion without affecting responses to carbachol. The relative molar potencies are shown in Table 1.

**Table 1**

| Compound | $CD_{100}$ µg/kg* i.v. mice | *Relative molar potency as GABA antagonist†* Frog spinal cord | Rat superior cervical ganglion |
|---|---|---|---|
| t-BPTBO | 25 | 3.5 | 4.2 |
| IPTBO | 140 | 1.0 | 1.0 |
| s-BPTBO | 100 | 0.47 | 1.0 |
| n-ProPTBO | 300 | 0.2 | 0.2 |
| n-BPTBO | 800 | 0.08 | 0.07 |
| PPTBO | 1200 | <0.01 | <0.01 |
| MPTBO | >4000 | — | <0.01 |
| Bicuculline | 200 | 0.5‡ | 1.0‡ |

* Mean values each determined from 8 groups of 4 mice.

† Determined by comparison with IPTBO within the same experiment. Mean values from 2–6 experiments for each compound.

‡ Bicuculline methochloride

t-BPTBO was the most potent antagonist and convulsant and was approximately four times more potent than bicuculline. The reasonably close agreement between the $CD_{100}$ values and the molar potencies as antagonists lends further support to the suggestion that the convulsant properties of the PTBO derivatives may be due to antagonism of the actions of synaptically-released GABA (Bowery *et al.*, 1976b).

We wish to thank Dr T.D. Inch for preparing t-BPTBO.

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## Influencing morphine self-administration in dependent rats

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Naive rats do not normally drink solutions of morphine, possibly because they are bitter tasting (to man) and possibly because of the post-ingestional effects of the drug. Rats can, however, learn to prefer such solutions (morphine HCl 0.5 mg/ml) to plain water if they are repeatedly 'forced' to drink them in order to relieve thirst. Once such preferences have been acquired, they are relatively stable and persistent and provide a useful animal model of dependence on morphine (Stolerman & Kumar, 1970). There are a number of conceptually distinct ways of trying to change patterns of behaviour that are maintained by pharmacological reinforcers. We report some experiments in rats with established preferences for morphine in which different 'treatment' procedures were compared for their ability to reduce or eliminate morphine-seeking behaviour.

Dependent rats ($n = 9$) which received injections of saline 2 h before their daily choice tests continued to ingest about 60% of their daily fluid intake in the form of morphine solution; this corresponded to a daily dose of approximately 20 mg/kg. Other rats ($n = 10$) were injected with morphine HCl (30 mg/kg), 2 h prior to their choice tests for 12 days and this treatment initially reduced the proportion of the daily fluid intake consumed as morphine solution from 63% to 43% ($P < 0.005$). Increasing the daily doses of injected morphine up to 120 mg kg day did not further reduce the proportional intake of the drug.

Rats ($n = 10$) treated with naloxone (initially naloxone HCl 1 mg $kg^{-1}$ $day^{-1}$ for 12 days, then increasing up to 8 mg $kg^{-1}$ $day^{-1}$) 30 min before choice trials showed little change in their patterns of morphine consumption. It is possible that naltrexone, which is also a relatively 'pure' opioid antagonist but with more prolonged actions, might have altered morphine intake. However, both morphine and naloxone were clearly effective in the range of doses used since they inhibited normal growth—the linear trends for the drugged rats' body weights differed significantly from the saline-injected group ($P < 0.01$). Thus, other important factors, apart from the need to relieve withdrawal symptoms or to obtain primary positive reinforcement, must be involved in sustaining morphine preferences.

When saccharin sodium (0.5 mg/ml) was added to the usual morphine solution and this novel taste was associated with an aversive state, naloxone-precipitated abstinence from morphine (cf. Pilcher & Stolerman, 1976), dependent rats ($n = 10$) subsequently markedly avoided this mixture in preference tests; they took about 5% of their fluid as drug solution. This aversion was long lasting and it did not generalize either to solutions of morphine alone ($P < 0.005$) or to saccharin alone ($P < 0.01$). Control (non-averted) subjects showed clear preferences for morphine + saccharin solutions and the preference for this now familiar taste was resistant to the aversion procedure.

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# Effects of tryptophan and portocaval anastomosis on activity and brain tryptophan metabolism

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Chronic portocaval anastomosis in the rat increases brain tryptophan and tyrosine, and thus 5-hydroxytryptamine (5-HT) (Curzon, Kantamaneni, Fernando, Woods & Cavanagh, 1975) and octopamine synthesis (James, Hodgman, Funovics & Fischer, 1976). These changes may be responsible for central disturbances in human liver failure (for review of evidence see Curzon & Knott, 1977). The role of tryptophan was investigated by giving the amino acid to rats with portocaval anastomosis.

Anastomosis or sham operation was performed on male Sprague-Dawley rats (mean body weight ± s.d. (*n*) 341 g ± 27 (28)) using the method of Funovics, Cummings, James, Shuman & Fischer (1975). Animals were caged singly 3 weeks after the operation and the experiment indicated in Table 1 was performed 4 weeks later.

Tryptophan (20 mg/kg i.p.) significantly decreased ambulation (but not rearing, turning, head-lifting or grooming) in sham-operated rats and significantly increased midbrain tryptophan (Table 1). 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) did not rise significantly. Rats with portocaval anastomosis also showed significantly less ambulation than sham-operated animals, and midbrain tryptophan, 5-HT and 5-HIAA were all significantly raised. Ambulation by anastomosed rats was not significantly decreased further by tryptophan, although midbrain tryptophan and 5-HT (but not 5-HIAA) rose significantly. Ambulation was significantly decreased and all biochemical measures significantly increased in tryptophan-treated anastomosed rats when compared with the sham-operated group given saline. Brain tryptophan rose more strikingly in anastomosed rats than in sham-operated animals given tryptophan.

These results are suggestive of an association between raised brain tryptophan, and/or 5-HT turnover, and central disturbances in liver failure.

**Table 1** Effects of tryptophan and portocaval anastomosis on activity and midbrain tryptophan metabolism

| | | | | | *Midbrain 5-HT* | |
|---|---|---|---|---|---|---|
| *Group* | *Rats* | *Injected* | *Ambulation* | *Tryptophan* | *µg/g wet wt* | *5-HIAA* |
| 1 | Sham (7) | 0.9% NaCl | 73±16 | 4.28±0.26 | 1.15±0.04 | 1.25±0.14 |
| 2 | Sham (7) | Tryptophan | 18± 8 | 5.73±0.41 | 1.26±0.03 | 1.63±0.16 |
| 3 | PCA (6) | 0.9% NaCl | 29±11 | 9.76±1.05 | 1.37±0.03 | 3.37±0.26 |
| 4 | PCA (7) | Tryptophan | 24± 7 | 16.23±1.92 | 1.59±0.06 | 4.58±0.56 |
| Group 1 v. Group 2 | | *p* | <0.005 | <0.05 | n.s. | n.s. |
| Group 1 v. Group 3 | | *p* | <0.05 | <0.001 | <0.01 | <0.001 |
| Group 3 v. Group 4 | | *p* | n.s. | <0.05 | <0.05 | n.s. |
| Group 1 v. Group 4 | | *p* | <0.05 | <0.001 | <0.01 | <0.001 |

PCA= portocaval anastomosis. Singly-housed rats were injected with 0.9% NaCl or L-tryptophan (20 mg/kg i.p.), placed in an open field and their behaviour observed by closed circuit television between 15 and 95 min after injection, a method based on that of Taylor (1976). They were then killed and determinations made as described by Curzon *et al.* (1975). Number of rats shown in parentheses. Results are expressed as mean ± s.e. mean. The number of areas entered (ambulation) were counted and values compared by the Mann-Whitney *U* test. Midbrain determinations were compared by Student's *t* test

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# Liver blood flow measurement with $^{85}$krypton clearance by portal venous and hepatic arterial routes of injection

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Using inert gas clearance for measurement of liver blood flow (LBF), workers in the past have concluded that there is incomplete mixing of blood from the portal vein and hepatic artery in the liver sinusoids (Hollenberg & Dougherty, 1966; Birtch, Casey & Zakheim, 1967). This was based on the finding that clearance of the gas was about 30% slower after injection into the hepatic artery than that after portal vein injection. It contradicts established anatomical views of the hepatic microcirculation and also throws doubt on the suitability of the inert gas clearance method for measurement of LBF.

This problem has been studied in the anaesthetized dog, using an $^{85}$Kr clearance technique for measuring LBF. Following its injection into the portal vein or hepatic artery, the beta emissions of the isotope were recorded using a Geiger-Müller tube positioned over the surface of the liver.

Variation seen in the anatomy of the hepatic artery suggested the possibility that standard hepatic arterial injection through the gastroduodenal artery might result in extrahepatic shunting of isotope to the portal vein, reduce the apparent clearance rate from the liver, and thus underestimate the true LBF. After hepatic arterial bolus injection of $^{85}$Kr, sequential samples of portal venous blood were analysed for radioactivity in 3 animals. In each case, significant activity was found in the portal vein 20 s later and remained for at least 3 minutes.

A dissection was designed which isolated the liver from local recirculation by arterial collaterals via the foregut to the portal vein. Following this, radioactivity was no longer found in portal venous blood after gastroduodenal artery injection, indicating that there had indeed been an extrahepatic shunt of $^{85}$Kr from the hepatic artery to the portal vein.

To determine the influence of such a shunt on LBF values obtained from clearance curves recorded after hepatic artery injection, clearances were performed in pairs by injection of $^{85}$Kr alternately into the portal vein and hepatic artery. Two groups of dogs were studied: Group A (9 dogs) had standard cannulations, while Group B (6 dogs) had similar cannulations plus the additional dissection described above.

The hepatic artery clearance in Group A was significantly slower than portal vein clearance ($P < 0.001$), the mean ratio LBF–hepatic artery route/LBF–portal vein route being $0.69 \pm 0.24$ (s.d.) in 42 pairs. In Group B, however, hepatic artery clearance was no longer significantly different from portal vein clearance ($P > 0.05$), with the mean ratio LBF–hepatic artery route/LBF–portal vein route $0.98 \pm 0.15$ (s.d.) in 37 pairs. There was a highly significant difference between the ratios of the two groups ($P < 0.001$).

These results show that when $^{85}$Kr reaches the liver through the hepatic artery with no extrahepatic shunt to the portal vein (Group B), it clears from the liver at the same rate as after injection by the portal venous route, thus confirming that hepatic arterial and portal venous blood mix completely in a common flow bed in the liver.

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# The pharmacokinetics of norethisterone in the rabbit and rat after systemic and oral administration: Effect of phenobarbitone

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Norethisterone is a synthetic progestogenic component of many oral contraceptive preparations. We have investigated the pharmacokinetics of norethisterone after intravenous (i.v.) and oral administration (85 μg/kg) to unanaesthetized female rabbits and i.v. and portal administration (50 μg/kg) to anaesthetized female rats. Blood samples were collected over 24 h from the marginal ear vein of the rabbit and over 2 h from the carotid artery of the rat.

Norethisterone was measured in plasma by radioimmunoassay using an antiserum raised in rabbits against norethisterone 3-(O-carboxymethyl) oxime bovine serum albumin. The antiserum was used at a dilution of 1 in 25,000.

In all experiments the plasma concentration-time curve was resolved into two exponential components. The values for the 'fast disposition' half life, the 'slow disposition' half life and the area under the curve (AUC) are listed in Table 1.

Norethisterone showed a marked first pass effect in both the rabbit and rat (Table 1). In the rabbit the AUC after oral administration was 53% of that after i.v. administration, whilst in the rat the AUC after portal administration was 32% of that after i.v. administration.

There is increasing evidence that enzyme induction may influence the efficacy of oral contraceptives. The effect of phenobarbitone on the previously determined pharmacokinetic parameters was therefore determined. Phenobarbitone was administered either in drinking water (1 mg/ml for 6 days) to rabbits or intraperitoneally (40 mg/kg twice daily for 4 days) to rats. Induction was indicated from a decrease in pentobarbitone sleeping time in treated rabbits from 3.6 to 1.1 h and from an increase (76%) of cytochrome P-450 in treated rats (0.43 ± 0.02 nmol/mg protein in controls; 0.75 ± 0.12 nmol/mg protein in treated rats). In the rabbit phenobarbitone had no significant effect on plasma norethisterone concentrations after i.v. administration but significantly reduced the plasma concentration after oral administration. In contrast, in the rat, phenobarbitone had little effect on plasma norethisterone concentrations despite the clear evidence of enzyme induction.

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**Table 1** Pharmacokinetic parameters of norethisterone in the rabbit and rat

| | *Intravenous* | *Oral* |
|---|---|---|
| Rabbit | | |
| 'Fast disposition' half life (h) | 0.4 ± 0.1 | 0.7 ± 0 1 |
| 'Slow disposition' half life (h) | 7.4 ± 2.1 | 12.5 ± 1 2 |
| AUC (h ng $ml^{-1}$) | 56.0 ± 2.9 | 30 9 ± 8.3 |
| Rat | | |
| 'Fast disposition' half life (min) | 5.1 ± 1.2 | 10 1 ± 2.3 |
| 'Slow disposition' half life (min) | 44 7 ± 6.6 | 72.0 ± 12 0 |
| AUC (min ng $ml^{-1}$) | 672 ± 129 | 223 ± 56 |

Each result is the mean ± s.e. mean of 5 experiments.

## Studies on the metabolic fate of 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) in man

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Many drugs are thought to exert their pharmacological effects by altering brain noradrenaline turnover. It would therefore be useful to have a non-invasive technique for the measurement of brain noradrenaline turnover in man. Measurement of noradrenaline levels in urine or plasma has not provèd to be a good index of central noradrenaline turnover, as little, if any, neurotransmitter leaves the brain without undergoing metabolism. A solution would be to assay a metabolite of noradrenaline formed only in the brain. Following the reports of Schanberg and co-workers (Schanberg, Schildkraut, Breese & Kopin, 1968a; Schanberg, Breese, Schildkraut, Gordon & Kopin, 1968b) considerable work has led to the belief that in most species, including man, one of the principal metabolites of noradrenaline in the brain is MHPG and further, that the sulphate conjugate of this metabolite has its origin almost exclusively in the brain.

We have therefore developed sensitive, specific stable isotope dilution assays for MHPG and for its sulphate and its glucuronide conjugates, utilizing computerized gas chromatography-mass spectrometry. MHPG and its sulphate conjugate, labelled specifically with deuterium atoms, were synthesized for use as internal standards. MHPG was isolated from 5 ml aliquots of urine by extraction with ethyl acetate while the two conjugates were measured after extraction on Amberlite XAD-2 resin, followed by chromatographic separation on Sephadex LH-20. Both MHPG and its sulphate could be converted directly to the MHPG *tris*-trifluoracetate derivative by treatment with trifluoracetic anhydride. The glucuronide was not cleaved by this reagent and hence did not interfere with determination of the sulphate.

Urinary excretion of the three metabolites in 10 normal volunteers over 24 h was as follows expressed as mg free MHPG (mean ± s.d.): MHPG 0.08 ± 0.03 mg, MHPG sulphate 1.05 ± 0.38 mg, MHPG glucuronide 1.30 ± 0.40 mg. Thus most MHPG in urine is conjugated, with almost equal quantities of the two conjugates present. Data from 4 patients with adrenal catecholamine-releasing tumours (phaeochromocytoma) revealed elevated urinary levels of all three metabolites. The ratio of glucuronide to sulphate, however, was 0.94–2.16 and thus similar to control values. In contrast, with lumbar CSF from 42 individuals an average of 84% of the MHPG was unconjugated. Preliminary results with pooled samples of CSF indicate that the remainder is present as the glucuronide. This finding is in conflict with previous reports in which conjugated MHPG in CSF was described as the sulphate (Schanberg *et al.*, 1968b).

It is concluded that MHPG can be conjugated with either sulphuric or glucuronic acids in the periphery whereas in the central nervous system conjugation is a minor pathway and appears to occur only with glucuronic acid.

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## Studies of the interaction of carbenoxolone sodium and warfarin sodium *in vivo*

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The potentiation of the anti-coagulant activity of warfarin by phenylbutazone is well known (Aggler, O'Reilly, Leong & Kowitz, 1967; O'Reilly & Levy, 1970). Investigations into the mechanism of potentiation both *in vivo* and *in vitro* suggest displacement of warfarin from plasma binding sites as the cause (O'Reilly & Levy, 1970; Jun, Luzzi & Hsu, 1972). We have previously demonstrated a difference in the binding sites on human serum albumin between phenylbutazone, warfarin and carbenoxolone using a technique *in vitro* (Gottfried, Parke, Sacra & Thornton, 1975). We suggested that an interaction, due to displacement from plasma protein binding sites, between carbenoxolone and warfarin, phenylbutazone and other protein-bound drugs was unlikely, having shown that carbenoxolone was binding at a different class of site to the other drugs.

In the present series of experiments we used a model *in vivo* to demonstrate the potentiation of warfarin anti-coagulant activity by phenylbutazone and the corresponding reduction in plasma half-life of warfarin. This model was then used to investigate any possible interaction of carbenoxolone with warfarin.

Fasted female Biorex Wistar rats (200 ± 10 g) were used in groups of six per treatment for each blood collection. All rats received 10 mg/kg warfarin sodium orally (2.5 ml/kg in distilled water) and at the same time half of the rats received subcutaneously (in 0.9% NaCl, 5 ml/kg) a dose of phenylbutazone (20 mg/kg) or carbenoxolone (40 mg/kg), i.e. at twice the molar equivalent of warfarin. Blood samples (3 ml) were obtained under ether anaesthesia at sacrifice using Hepes/citrate buffer (0.13 M trisodium citrate; 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulphonic acid) 0.1 ml per ml of blood collected.

A one-stage prothrombin time was determined in duplicate for each rat using rabbit brain thromboplastin (Diagnostic Reagents Ltd) and warfarin concentrations were measured by spectrofluorimetry (Corn & Berberich, 1973).

No changes in prothrombin times from normal (17.0 ± 0.3 s), were seen at 8 h after warfarin treatment but at 24 h the prothrombin time was increased to 79.6 ± 1.7 s and 87.5 ± 3.5 s in the two experiments. Phenylbutazone, at a dose of twice the molar equivalent of warfarin, increased the time to 109.7 ± 7.1 s ($P < 0.005$, t-test) whilst carbenoxolone, at the twice molar equivalent, showed no significant change, 77.2 ± 3.5 seconds. Carbenoxolone and phenylbutazone had no inherent activity on prothrombin times at the dose regimens used.

The plasma half-life of warfarin, in the absence of other drugs, was found to be 8.4 and 8.5 h. Phenylbutazone reduced this to 2.3 h whilst carbenoxolone showed no change at 9.2 hours.

These findings show that carbenoxolone sodium *in vivo* does not potentiate or displace warfarin sodium and are in agreement with our earlier suggestions from studies *in vitro* (Gottfried *et al.*, 1975).

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## Rat brain iso-renin

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The components of the renin-angiotensin system have been demonstrated within the brain and they have been shown to be implicated in physiological control of water and sodium balance (Fitzsimons, 1975). Recently a brain iso-renin, distinct from the renal enzyme has been demonstrated in the dog and rat brain (Ganten, Hutchinson, Schelling, Ganten & Fischer, 1976). It has also been tentatively suggested that centrally-acting hypotensive drugs may act via brain iso-renin. In order to test this hypothesis and to study in more detail the physiological role of brain iso-renin, a method has been developed which enables small amounts of enzyme present in rat brain to be measured reliably.

Rat brain extracts were incubated at 37°C in the presence of substrate prepared from nephrectomized dogs according to the method of Hass, Goldblatt, Gipson & La Vera Lewis (1966). The angiotensin produced was adsorbed on to an ion exchange resin, and subsequently eluted from the resin at the end of the incubation period. The activity of this pressor material was estimated against synthetic angiotensin II on the pithed rat blood pressure preparation. The use of (Sar$^1$, Ala$^8$)-angiotensin II, a competitive antagonist of angiotensin II (Pals, Masucci, Denning, Sipos & Fessler, 1971), completely abolished the biological activity of the material eluted from the resin. Control experiments showed that the recovery of synthetic angiotensin II amide from the resin after a 24 h incubation period under the experimental conditions was 77 ± 3% (mean ± s.e. mean, $n = 15$). No pressor activity was detectable in zero time incubation samples or in samples incubated at 4 °C.

The pH optimum of the rat brain iso-renin dog substrate system was noted to be in the region of pH 4.4 to 4.8, and the apparent Michaelis constant $K_m$, of the system about 0.5 µM (expressed as angiotensin II). With the level of substrate used for routine assay, the reaction appeared to be first order and the generation of angiotensin was linear for over 24 hours. Using this technique brain iso-renin activity in normal male Wistar rats has been found to be in the range 90–100 ng Angiotensin II g brain$^{-1}$ hour$^{-1}$. This level of activity should be sufficient to allow an investigation of regional differences in brain renin activity and of any changes induced by therapeutic agents or by changes in physiological status.

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# The role of transmembrane calcium flux in the adrenergic response of the isolated frog heart

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Kunos & Szentivanyi (1968) have reported that the response of the isolated winter frog heart to catecholamines alters with temperature in a manner consistent with a direct, temperature-dependent interconversion of α- and β-adrenoceptors. Buckley & Jordan (1970) postulated the coexistence of both receptor types whose activation was achieved at different temperatures. In support of this postulate, the relative potencies of adrenaline and isoprenaline were reversed when heart temperature was lowered. The aim of the present study was to further characterize the adrenergic response at 23 °C and 6 °C in the winter frog heart.

Isolated hearts were suspended in frog Ringer's solution at 6 °C or 23 °C and treated with 1 µM [*N*-(3,4-dimethoxyphenethyl)-*N*-methylamino]-α-(3,4,5-trimethoxyphenyl)α-isopropylvaleronitrile hydrochloride (D600 hydrochloride) for 30 minutes. Rate and contraction amplitude of the heart decreased at both temperatures. The greater rate decreases at 23 °C or amplitude reductions at 6 °C did not differ sufficiently to indicate temperature-mediated dissociation of these effects. For this reason, decrease in performance was measured as percentage change in work output (amplitude × rate). The reduction in work output obtained with D600 at 23 °C was much greater than at 6 °C (see Table 1). Subsequent treatment with isoprenaline at 23 °C resulted in restoration of control values while adrenaline only partly overcame the reduction. In hearts studied at 6 °C, adrenaline was now more effective than isoprenaline.

It has been proposed that D600 inhibits excitation-contraction coupling by selective blockade of calcium transmembrane flux into excited myocardial fibres (Fleckenstein, 1971; Kohlhardt, Bauer, Krause & Fleckenstein, 1972). D600 has previously been used to differentiate between the inotropic actions of isoprenaline and phenylephrine in isolated guinea-pig ventricle (Ledda, Marchetti & Mugelli, 1975). The interactions described above would indicate that at

**Table 1** Percentage changes in work output (± s.e. mean) produced by D600 ($1 \times 10^{-6}$ M) and modified with adrenaline ($1.2 \times 10^{-5}$ M) or isoprenaline ($1.2 \times 10^{-5}$ M)

| | *D600* | *ADR* | *ISOP* |
|---|---|---|---|
| 23 °C | −65.3 ± 4.6 (6) | | +9.5 ± 10.00 |
| | −67.8 ± 3.6 (6) | −31.5 ± 13.3 | |
| 6 °C | −12.5 ± 2.5 (4) | | −4.0 ± 7.4 |
| | −17.7 ± 1.0 (9) | +22.4 ± 6.3 | |

23°C the considerable reduction in calcium flux caused by D600 is more effectively antagonized by isoprenaline than by adrenaline. It is possible that at 6°C the greater potency of adrenaline may reflect the development of an alternative pathway for the adrenergic response. In addition, calcium transmembrane flux may contribute less to the contractile response at 6°C since D600 effects a much smaller work output reduction.

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## Responses of the sympathetically-innervated hepatic arterial vascular bed of the dog to intra-arterial injections of dopamine

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The hepatic arterial vascular responses to intra-arterial (i.a.) noradrenaline and adrenaline have been studied in the dog (Andrews, Hecker, Maegraith & Ritchie, 1955; Richardson & Withrington, 1976a, 1977) and have been shown to involve stimulation of both $\alpha$- and $\beta$-adrenoceptors. The third naturally occurring catecholamine, dopamine, has been shown to produce both vasodilatation and vasoconstriction in the dog (Yeh, McNay & Goldberg, 1969; Sampson, Scroop & Louis, 1974), effects which may be due to stimulation of both $\alpha$- and $\beta$-adrenoceptors in addition to a specific dopamine receptor. The responses of the sympathetically-innervated hepatic arterial bed of the dog to dopamine, noradrenaline and adrenaline have been compared and the receptors mediating the dopamine effects examined.

In 6 chloralose-urethane anaesthetized dogs (Richardson & Withrington, 1976c), weighing 11.9 ± 1.9 kg (mean ± s.d.), under control conditions the hepatic arterial blood flow was 183.6 ± 46.8 ml/min (mean ± s.d.), and the perfusion pressure 121.3 ± 13.4 mmHg; the calculated hepatic arterial vascular resistance (HAVR) was 0.72 ± 0.28 mmHg $ml^{-1}$ min, or expressed in terms of the liver weights (272.0 ± 44.4 g), 1.87 ± 0.63 mmHg $ml^{-1}$ min 100 grams.

Dopamine was injected i.a. over the range 100 ng to 1 mg to construct 8 dose-response curves in 6 experiments: each injection produced an initial increase in calculated HAVR (vasoconstriction) followed by a secondary fall in HAVR (vasodilatation). The threshold for the vasoconstrictor response was 5–50 µg, the mean dose required to double the HAVR, $6.2 \times 10^{-7}$ mol, being much greater than the corresponding doses for noradrenaline, adrenaline and phenylephrine (1.1, 2.7 and $6.9 \times 10^{-8}$ mol respectively). The threshold for the vasodilator response (0.1–10 µg) was lower than that for the vasoconstrictor effect, the maximum reduction in HAVR of 25.7 ± 2.5% (mean ± s.e. mean) occurred at between 10 and 200 µg in different experiments.

These responses to dopamine were similar to those to adrenaline and noradrenaline, the secondary vasodilator effects of which have been shown to be due to $\beta$-adrenoceptor activation (Richardson & Withrington, 1976b, 1977).

In three experiments, the dose-response curves for both the vasoconstrictor and vasodilator responses to dopamine were constructed before and after propranolol (0.25 mg/kg i.v.). In common with adrenaline and noradrenaline, the vasoconstrictor dose-response curve to dopamine was shifted to the left, but in contrast to adrenaline and noradrenaline, the vasodilator dopamine dose-response curve was also shifted to the left, and at one dose level of dopamine ($5.3 \times 10^{-8}$ mol), this potentiation was statistically significant ($P < 0.05$, paired $t$-test).

These observations suggest that the hepatic arterial dilator responses to dopamine do not involve the same receptors as those which induce vasodilatation to adrenaline or noradrenaline, a conclusion supported by preliminary experiments with haloperidol (1.0 mg/kg i.v.) which markedly antagonized the hepatic arterial dilator effects to dopamine without affecting those to adrenaline or noradrenaline. Moreover the vasoconstrictor responses to both dopamine and phenylephrine were antagonized by haloperidol.

These results accord with the view that dopamine causes hepatic arterial vasoconstriction by stimulating $\alpha$-adrenoceptors, and vasodilatation by activating specific dopamine receptors.

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## UK-14,275, a novel orally-active cardiac stimulant

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UK-14,275, 1-butyl-3-[1-(6,7-dimethoxyquinazolin-4-yl)piperidin-4-yl] urea, was selected for further evaluation from a structurally novel series of cyclic nucleotide phosphodiesterase inhibitors that were found to stimulate preferentially the force as opposed to the frequency of cardiac contraction.

In spontaneously beating guinea-pig atria, UK-14,275 ($10^{-6}$ to $10^{-4}$ M) displayed dose-related positive inotropic activity coupled with negative chronotropic activity. In this respect it resembled ouabain ($10^{-7}$ to $10^{-6}$ M) but differed from isoprenaline ($10^{-9}$ to $10^{-7}$ M) and theophylline ($5 \times 10^{-5}$ to $5 \times 10^{-4}$ M) which increased force and frequency of contraction in parallel.

Intravenous injections (0.1 to 1.0 mg/kg) or infusions (0.05 to 0.5 mg $\text{kg}^{-1}$ $\text{min}^{-1}$ for periods up to 1 h) of UK-14,275 produced dose-dependent positive inotropic effects in anaesthetized and conscious dogs. Increases in heart rate were produced, but were markedly less than those evoked by an equally inotropic dose of isoprenaline.

Oral administration of UK-14,275, at doses above 5 mg/kg, evoked positive inotropic effects lasting from 3 to 6 h coupled with small increases in heart rate.

UK-14,275 was twenty times more potent than theophylline as an inhibitor of beef heart cyclic AMP phosphodiesterase *in vitro*, and was shown to increase tissue cyclic AMP levels in electrically driven guinea-pig left atria at a concentration of $5 \times 10^{-5}$ M. In addition, UK-14,275 ($5 \times 10^{-5}$ M) potentiated both the inotropic response and the increase in tissue cyclic AMP levels evoked by isoprenaline in driven guinea-pig left atria. In contrast to isoprenaline, UK-14,275 ($10^{-6}$ to $5 \times 10^{-4}$ M) did not affect the activity of guinea-pig heart adenyl cyclase. Furthermore, unlike ouabain, UK-14,275 ($10^{-8}$ to $10^{-4}$ M) had no effect on $Na^+$–$K^+$ ATPase activity.

In driven cat left atria, propranolol ($6.6 \times 10^{-8}$ M) reduced the maximum inotropic response to UK-14,275. Attenuation of the inotropic responses to UK-14,275 was also seen in tissues which had been pretreated with syrosingopine or reserpine. This partial dependence of the activity of UK-14,275 on catecholamines is compatible with phosphodiesterase inhibition, since catecholamines increase the rate of cAMP synthesis.

UK-14,275 is a novel, orally active inotropic agent which preferentially increases force as opposed to frequency of contraction. This unusual property is shared only by the cardiac glycosides to which it bears no structural or mechanistic resemblance. Studies to date suggest that the major part of its activity is mediated *via* the inhibition of phosphodiesterase. Clinical evaluation of UK-14,275 as an agent for the treatment of congestive heart failure is in progress.

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# Histamine-induced changes in heart rate in anaesthetized cats

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Histamine increases the rate of beating of isolated cardiac preparations due to interaction with histamine $H_2$-receptors (Black, Duncan, Durant, Ganellin & Parsons, 1972). Histamine also increases heart rate in anaesthetized animals (e.g. Tucker, Weir, Reeves & Grover, 1975; Powell & Brody, 1976) although the response has not been characterized. Tachycardia due to interaction with histamine $H_2$-receptors has not been demonstrated *in vivo*. Doses of histamine which increase heart rate also lower blood pressure and can release catecholamines from chromaffin tissue (Burn & Dale, 1926), both mechanisms which might elicit tachycardia independent of histamine $H_2$-receptors. Experiments have been made to characterize the mechanism of tachycardia after administration of histamine to anaesthetized cats.

Cats, of either sex, were anaesthetized by intraperitoneal injection of chloralose (60 mg/kg) and urethane (700 mg/kg). The trachea was cannulated. Blood pressure was measured from one femoral artery and heart rate measured using a rate meter triggered by the blood pressure pulse. Drugs were administered via catheters in each brachial vein.

Histamine caused dose-dependent depressor responses and tachycardia over the dose range $1 \times 10^{-9}$ to $1 \times 10^{-7}$ mol/kg. Tachycardia persisted after treatment with mecamylamine (5 mg/kg), to block autonomic ganglia and prevent reflex increases in heart rate associated with the depressor responses to histamine.

Tachycardia caused by histamine up to $1 \times 10^{-7}$ mol/kg, in ganglion-blocked cats could be reduced or abolished by treatment of cats with either propranolol (1 mg/kg) or mepyramine (5 mg/kg) suggesting that the tachycardia caused by these doses of histamine was due to release of catecholamines from chromaffin tissue, a histamine $H_1$-receptor phenomenon (Emmelin & Muren, 1949). Increasing the dose of histamine to $1 \times 10^{-6}$ mol/kg in mepyramine-treated cats restored tachycardia. This response to larger doses of histamine was refractory to propranolol or further doses of mepyramine but antagonized by metiamide, 0.5 mg kg$^{-1}$ min$^{-1}$ indicating that these large doses of histamine can cause tachycardia by interaction with histamine $H_2$-receptors.

These experiments indicate that histamine can cause tachycardia in anaesthetized cats independent of reflex responses to falls in blood pressure. After low doses of histamine this response is due to release of catecholamines whereas larger doses of histamine can also cause tachycardia by interaction with histamine $H_2$-receptors.

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# The effects of L9394 on adrenergic transmission in the cat spleen

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L9394 (2-ethyl-3-(4-$\gamma$-di-$n$-butylaminopropoxybenzoyl)-indolizine hydrochloride) has, in the dog, similar haemodynamic effects to amiodarone (Charlier, Bauthier & Richard, 1976). Amiodarone is known to produce a dose-dependent reduction in the overflow of transmitter from the isolated blood perfused cat spleen following nerve stimulation at 30 Hz yet has no effect on release induced by tyramine (Bacq, Blakeley & Summers, 1976). We have examined the effects of L9394 upon transmitter release and overflow in the isolated blood perfused cat spleen (for methods and experimental plan see Bacq *et al.,* 1976). Each dose of the drug was added to the blood perfusing the spleen and allowed to act for 30 minutes. L9394 produced a dose-dependent decrease in transmitter overflow from the spleen following trains of 200 stimuli at 30 Hz ($r=0.97$ d.f.$=9$). The $ED_{50}$ for this effect was 0.24 mg/g wet wt. of spleen ($CL_{95}$ 0.19–0.32). The response of the spleen to nerve stimulation was reduced in a parallel manner to the reduction in overflow. In direct contrast to this effect on stimulation evoked release of transmitter, L9394 (0.9 mg/g wet wt. spleen) had no effect on the release induced by tyramine of [$^3$H]-(−) noradrenaline pre-loaded into the spleen by infusion 30 min earlier. After L9394 the intra-arterial injection of tyramine (50 µg) released 106.5% of the control release of label but stimulation-evoked release was reduced to 3.7% of control (1 experiment).

L9394 (0.5 mg/g wet wt. spleen) did not alter the ability of the spleen to take up [$^3$H]-(−) NA from an intra-arterial infusion at 345 ng/minute. At steady state control spleens took up 45.5 ± 1.6% ($n=20$) of the infusion compared with 51.1 ± 5.3% ($n=8$) for spleens treated with L9394.

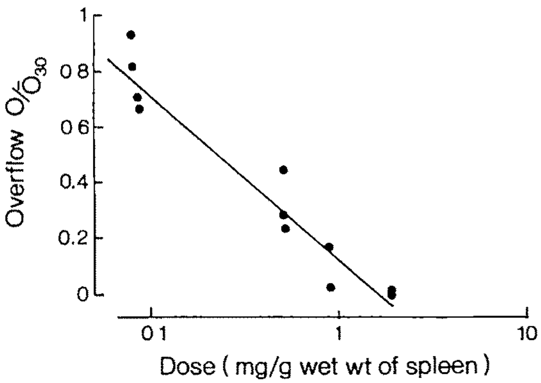

**Figure 1** The effect of L9394 on overflow of transmitter following 200 stimuli at 30 Hz. Overflow (O) is expressed in terms of the mean pre-drug value ($\bar{O}_{30}$).

L9394 therefore not only resembles amiodarone in its haemodynamic profile but has similar effects on adrenergic transmission in the spleen.

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## Plasma noradrenaline levels as a measure of noradrenaline release

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In order to monitor noradrenaline (NA) release over extended periods, cannulae were inserted into the right atrium of rats through the superior vena cava under anaesthesia. Tubing connected to the cannula runs subcutaneously and is brought to the surface in the midscapular region. Rats were allowed to recover from the anaesthesia and were kept on a standard laboratory diet. Blood samples of 0.25 ml were withdrawn and replaced with an equal volume of sterile saline. The blood was put into heparinized tubes containing 1.6 mg glutathione/ml blood and centrifuged; the serum was diluted 1:4 with distilled water and stored deep frozen. The NA content of the samples was assayed using a radioenzymatic method (Hörtnagl, Benedict, Grahame-Smith & McGrath, 1977). The NA content of plasma in untreated rats was $1.88 \pm 0.08$ ng/ml ($n=16$, C.V.$=0.58$). When desmethylimipramine (DMI; 10 mg/kg) and normetanephrine (NMN; 50 mg/kg) were given in a single intraperitoneal injection, to block neuronal and extraneuronal NA uptake mechanisms respectively, plasma NA rose to $5.26 \pm 0.51$ ng/ml ($n=9$). In order to discover whether there were changes related to the time of day, plasma NA was measured in a group of rats at hourly intervals between 1100 and 1700 hours. There were no statistically significant variations in plasma NA level throughout this period and the mean coefficient of variation for the 6 h period was 0.50, similar to that for the control value, which represents individual variation. Experiments were next carried out to measure the effect of the administration of NA. Plasma NA was measured at various time intervals after the intravenous administration of 100 μg/kg of NA; calculations carried out on the basis of these measurements showed that 1 min after NA administration only 4% of the administered dose was found in the circulation. There followed an approximately exponential decline in plasma NA levels with a $T_{\frac{1}{2}}$ of 1.5–2 minutes. In order to compare the fate of exogenous and endogenous NA rats were subjected to a 1 min swim stress. Blood samples taken before and at the end of the swim stress (water temperature 17°C) showed a rise in plasma NA from $2.33 \pm 0.13$ ng/ml to $9.44 \pm 2.06$ ng/ml. After the termination of the swim stress plasma NA levels fell steeply within the first 2 minutes. This initial rate of decline was affected very little by the administration of DMI (10 mg/kg) and NMN (50 mg/kg).

These preliminary experiments suggest that plasma NA in rats shows little variation between individuals or with time of day; although only a fraction of the released NA escapes uptake and reaches the blood stream, even short-lasting changes in release rate are reflected in plasma NA levels. NA in plasma has a very short half-life and even in the presence of DMI and NMN mechanisms for the rapid removal of NA remain.

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## Effects of uptake blockade on cardiac responses of anaesthetized dogs to isoprenaline, noradrenaline, and sympathetic nerve stimulation

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The greater muscle mass of the ventricular myocardium relative to that of the atria suggests that extraneuronal uptake (uptake 2) of catecholamines may perform a more substantial role in the termination of inotropic rather than chronotropic responses of the heart to catecholamines. In contrast, the higher density of sympathetic innervation in the sino-atrial and atrial tissues raises the possibility that neuronal uptake (uptake 1) may be more important in the termination of chronotropic than inotropic responses.

Accordingly, we have investigated the effects of differential and combined blockade of uptake 1 and uptake 2 on cardiac responses of anaesthetized dogs to (±)-isoprenaline, (−)-noradrenaline and electrical stimulation of the left ansa subclavia nerve.

Beagle dogs of either sex were studied (10.5–16.0 kg) under sodium pentobarbitone anaesthesia (30 mg/kg i.v.). The animals were artificially respired and arterial $PO_2$, $PCO_2$ amd pH were checked repeatedly during the experiments and maintained within normal physiological limits. Both cervical vago-sympathetic nerve trunks were sectioned and the chest was opened in the mid-line. Left ventricular pressure, dp/dt max., aortic blood pressure and heart rate were obtained by use of Millar catheter tip pressure transducers, an appropriate differentiator and ratemeter and registered on a Devices M19 8-channel recorder.

Cocaine hydrochloride (5 mg/kg i.v., and 1 mg/kg every 45 min) was used to block uptake 1 (Trendelenburg, 1959), and (±)-metanephrine (40 μg $kg^{-1}$ $min^{-1}$ i.v.) to block uptake 2 (Burgen & Iversen, 1965). Dose-response curves to i.v. bolus injections of isoprenaline (50 ng/kg–1 μg/kg) and noradrenaline (100 ng/kg–2 μg/kg) and frequency-response curves (supramaximal voltage, 5 ms pulse width, 1.0–20 Hz) of electrical stimulation of the left ansa subclavia nerve established under control conditions were compared with those obtained during individual and combined administration of the uptake antagonists. Statistical testing was by analysis of variance.

Cocaine potentiated responses to noradrenaline or nerve stimulation, but did not affect those to isoprenaline. Metanephrine potentiated positive inotropic responses to all doses of isoprenaline ($P<0.05$) and positive chronotropic responses were enhanced at doses $\geqslant$100 ng/kg. Positive inotropic responses to noradrenaline were enhanced significantly at doses $\geqslant$250 ng/kg, and chronotropic responses at doses $\geqslant$1 μg/kg. Metanephrine did not affect positive inotropic responses to sympathetic nerve stimulation of any frequency and cardiac responses to noradrenaline or nerve stimulation obtained after administration of cocaine were not further modified by the concurrent administration of metanephrine. Similarly, responses to isoprenaline in the presence of both cocaine and metanephrine were similar to those obtained in the presence of metanephrine alone, but subsequent administration of cocaine to those preparations already receiving metanephrine still potentiated cardiac responses to noradrenaline or nerve stimulation.

Our results confirm the known effects of cocaine on cardiovascular responses to noradrenaline and sympathetic nerve stimulation and establish that metanephrine enhances cardiac responses to isoprenaline *in vivo*. However, plots of dp/dt max. against heart rate demonstrate that cocaine preferentially enhances chronotropic responses to noradrenaline, but that metanephrine does not discriminate between inotropic and chronotropic effects of isoprenaline.

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## Studies on the rebound hypertension after clonidine withdrawal in conscious hypertensive cats, rats, and dogs

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Clonidine is a potent antihypertensive agent which is known to act on central cardiovascular sites and its effect on these centres is thought to be due to stimulation of central $\alpha$-adrenoceptors (Schmitt, Schmitt & Fénard, 1973; Finch, 1974). The reports of a severe rebound rise in blood pressure if the drug is withdrawn abruptly remains a problem in the treatment of patients and also its mechanism is poorly understood (Hunyor, Hansson, Harrison & Hoobler, 1973).

In both spontaneous hypertensive and normotensive rats ($n=8$), clonidine ($2\times0.2$ mg/kg s.c. for 14 days) produced a marked fall in blood pressure measured by the indirect tail cuff method: on withdrawal of clonidine (15 days), the blood pressures returned to normal.

In conscious renal hypertensive cats ($n=6$) with blood pressure recorded from cannulae chronically implanted into the thoracic aortae (Finch, 1974), treatment with clonidine ($3\times0.025$ mg p.o. for 10 days) also produced a sustained fall in mean blood pressure (40 mmHg). However, after completion of the clonidine treatment (day 11), 4 cats exhibited a rebound hypertension (20–30 mmHg) above pretreatment levels and also a marked tachycardia.

In conscious renal hypertensive dogs ($n=5$) with blood pressure recorded from chronically implanted arterial cannulae, clonidine pretreatments (3 x 0.15 mg p.o. for 4 or 10 day periods) produced sustained falls in blood pressure and marked bradycardia. In the 3 mongrel dogs a rebound hypertension (40–80 mmHg) and tachycardia was observed after completion of either treatment (day 5 or 11) but rebound hypertension was not observed in beagle dogs ($n=2$). The mechanism of this rebound hypertension on clonidine withdrawal remains to be investigated but it would seem that two experimental models are available for this study.

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# The effects of $\beta$-adrenoceptor blockade on the development of deoxycorticosterone acetate (DOCA) hypertension in the dog

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Salt-loading in dogs with partial nephrectomy has been shown to produce hypertension which is dependent upon an elevation of cardiac output (Coleman & Guyton, 1969; Cowley & Guyton, 1975) followed by autoregulation and an increase in vascular resistance. It seemed possible therefore to examine this process during the development of DOCA hypertension. We have produced a model of DOCA hypertension in the dog in which it was possible to evaluate the effects of $\beta$-adrenoceptor blockade during the developmental stages to determine the role of changes in cardiac output.

Male beagle dogs (13.0–15.5 kg) were chronically implanted with carotid artery and jugular vein vinyl catheters from which blood pressure (BP) and cardiac output (CO dye-dilution) measurements were made. Plasma volume (PV) was measured by the Evans Blue method and extracellular fluid volume (ECFV) by determination of the thiocyanate space. Several weeks after unilateral nephrectomy, DOCA (1.0 g) was implanted subcutaneously in 5 dogs, and 3 days later they were given a drinking solution containing 1.0% NaCl, 0.25% KCl and 0.25% sugar *ad libitum* for a further 15 days. At the end of this period, drinking water containing 0.25% KCl and 0.25% sugar was provided. A group of 4 dogs similarly treated were given atenolol 150 mg twice daily during the saline drinking period to investigate the effect of $\beta$-blockade on the development of the hypertension.

In dogs receiving DOCA and saline alone there was an increase over 7 days in systolic BP 149±3.3 to 182±9.0 mmHg ($P<0.01$) and in diastolic BP 86±1.9 to 111±4.6 mmHg ($P<0.01$). These changes were sustained and were accompanied by an initial reduction in heart rate (HR) from 81±4.3 to 60±4.2 bts/min which gradually returned to control values. There was a small increase in CO of approximately 0.48 l/min after 7 days and total peripheral resistance (TPR) increased by 30% after 3 days but these changes were not statistically significant. PV increased from 917±26 to 1116±57 ml and ECFV 4.98±0.20 to 5.94±0.581 ($P<0.05$) at day 7 but returned to control values at day 14.

In the group receiving atenolol along with DOCA and saline, the BP rose as it did in the last group from 138±4.8 to 189±3.8 mmHg systolic ($P<0.01$) and 85±4.5 to 113±4.3 mmHg diastolic ($P<0.01$). HR fell from 90±8.9 to 50±11.6 bts/min (day 14), CO was reduced during the first 7 days from 2.30±0.17 to 1.91±0.29 l/min ($P<0.05$). This effect was more pronounced by day 14 when CO was 1.63±0.07 l/min ($P<0.01$, TPR increased progressively over 14 days from 3696±242 to 6289±356 dynes sec cm$^{-1}$ ($P<0.002$). The changes in PV and ECFV showed a small upward trend which was not statistically significant.

In control experiments in 4 normotensive dogs, atenolol 150 mg p.o. twice daily produced a small fall in BP from 145±7.0 to 130±5.0 mmHg systolic and 80±7.0 to 58±4.0 mmHg diastotic ($P<0.01$) over a period of 24 days.

In conclusion therefore DOCA and saline produced an elevation in blood pressure which was associated with an increase in both cardiac output and peripheral resistance and an expansion of plasma and extracellular fluid volumes. $\beta$-Adrenoceptor blockade did not prevent the development of DOCA/saline hypertension in spite of the fact that it prevented a rise in cardiac output.

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# The presence of prostaglandin-like material in carrageenin induced rat hind paw inflammation

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The involvement of prostaglandins (PGs) in the delayed phase of carrageenin hind paw oedema (Di Rosa, Giroud & Willoughby, 1971) is generally accepted. Direct evidence for this assumption is based on bioassay of exudate, squeezed out of amputated inflamed paws (Willis, 1969). However, data on non-inflamed paws or on time of collection were not reported. These omissions are important since tissue damage and concomitant platelet aggregation are likely to produce PGs. To avoid this pitfall we used a coaxial perfusion technique (Rocha e Silva & Antonio, 1960) to collect part of the exudate.

Male Wistar rats (180–250 g) were anaesthetized with urethane (25%) – chloralose (2%). Polythene cannulae (diameter 3 mm) were inserted, subcutaneously, through a small incision in the lateral skin of the tarsus, and pushed into the subplantar region. Perfusion with 6% dextran in sterile pyrogen-free saline was carried out with an innercannula (diameter 1 mm) extending 3–4 mm. After removal of traces of blood (30 min, 4 ml/h), the perfusion was continued (30 min, 2 ml/h) to obtain a basal measurement of PG-like activity (PGL). Thereafter, either sodium-carrageenin (0.1 ml, 1%) or saline was injected subplantarly. Biological activities in perfusates, collected (30 min, 2 ml/h) at different times after these injections, were tested directly on a cascade of isolated tissues (rat stomach, rat colon and cat jejunum) in the presence of appropriate antagonists. No serotonin (<1 ng/paw) or bradykinin-like activities (<0.5 ng/paw) were detectable. The results concerning PGL are given in Table 1.

**Table 1** Collection of prostaglandin-like activity (PGL) from rat hind paws before (–30 min) and after treatment with carrageenin or saline

| *Treatment Perfusion time* | *Saline PGL (ng $PGE_2$/paw)* | *Carrageenin PGL (ng $PGE_2$/paw)* |
|---|---|---|
| –0.5–0 h | <1.0 (8) | <1.0 (10)<br>1.1 (1) |
| 1–1.5 h | <1.0 (2) | <1.0, 1.3 (2) |
| 2–2.5 h | <1.0 (2) | 2.0 (1) |
| 4–4.5 h | <1.0 (2) | 2.9 ± 0.3 (4)* |
| 6–6.5 h | <1.0 (2) | 3.3 ± 1.2 (4)* |

The values are expressed as ng $PGE_2$/paw since the efficiency of the perfusion in removing PGs from the exudate is difficult to estimate. All values are means ± s.e. mean. The numbers of observations are given in brackets. * $P<0.05$, when compared with zero-time controls. (Mann-Whitney *U* test, one-tailed).

These data directly support the assumption (Di Rosa *et al.*, 1971) that PGs are present in the late phase of carrageenin oedema. However, the total amount *in situ* is probably higher. Since PGL was undetectable in saline pretreated paws it is unlikely that tissue irritation by the prolonged presence of cannulae is a source of PGL under these conditions. Similar results were obtained with lipid extracts from perfusates, collected at 4 h after carrageenin or saline injection when cannulae were installed immediately after sacrificing the rats or immediately after indomethacin pretreatment (2 mg/kg i.v.) of rats under pentobarbitone anaesthesia.

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# Potentiation of bradykinin-induced exudation following intradermal injection of particulate or colloidal materials in the rabbit: evidence for prostaglandin release and action in inflammation

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Prostaglandins, which have little effect on vascular permeability when injected alone into rabbit or guinea-pig skin, potentiate the local exudation produced by bradykinin and histamine (Williams & Morley, 1973). This potentiating activity of prostaglandins correlates with their vasodilator activity (Williams, 1976a). The present study is concerned with the source and nature of possible endogenous potentiating substances in inflammatory reactions.

Plasma exudation and blood flow changes were measured in rabbit skin using $^{131}$I-albumin and $^{133}$Xe, as previously described (Williams, 1976b). It was observed that an intradermal injection of *B. pertussis* ($2.5 \times 10^8$ organisms/site) greatly potentiated the exudation produced by a following injection of bradykinin (500 ng/site) into the same site. Similar potentiation of responses to bradykinin (or histamine) was observed using zymosan, glycogen, antigen-antibody complexes, and carrageenin. No potentiation was observed with bradykinin/*B. pertussis* mixtures. Potentiation increased as the interval between the two injections was increased up to 1 h, but decreased with longer intervals (Figure 1). The small exudation produced by *B. pertussis* alone, followed a similar time-course.

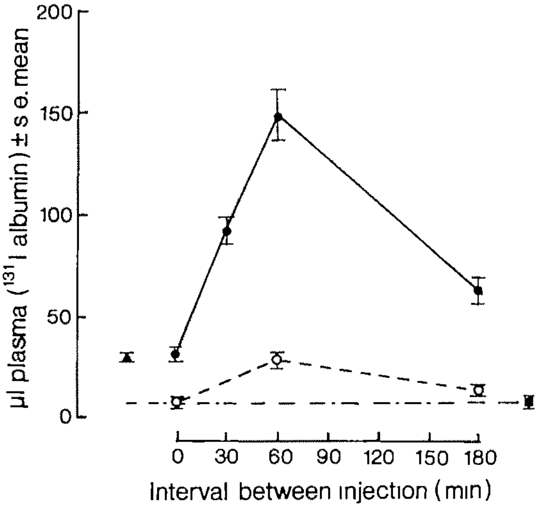

**Figure 1** Time-course of potentiation of bradykinin (500 ng)-induced exudation by a preceding intradermal injection of *B. pertussis* ($2.5 \times 10^8$ organisms) in rabbit skin. ● 1st injection of *B. pertussis*/2nd injection of bradykinin, ▲ saline/bradykinin, ○ *B. pertussis*/saline, ■ saline/saline. Abscissa is interval between 1st and 2nd injections. Exudation was measured for 30 min following 2nd injection. $n = 6$.

The potentiation time-course suggested that an accumulation of haematogenous cells was a prerequisite. This was investigated in polymorphonuclear (PMN-) leucocyte-depleted rabbits using nitrogen mustard (1.75 mg/kg i.v., 4 days before experiment). A marked reduction in *B. pertussis* potentiation (1 h interval) was observed in these animals with no significant reduction in PGE$_2$ potentiation; (exudation, nitrogen mustard treated: bradykinin = 26.8 ± 5.4 μl, *B. pertussis* = 0.0 ± 1.1 μl, *B. pertussis*/bradykinin = 46.7 ± 8.9 μl, bradykinin + PGE$_2$ (100 ng) = 81.4 ± 17.7 μl; controls: bradykinin = 33.5 ± 7.8 μl, *B. pertussis* = 20.0 ± 4.9 μl, *B. pertussis*/bradykinin = 126.2 ± 19.5 μl, bradykinin + PGE$_2$ = 90.9 ± 14.4 μl; $n = 7$ rabbits/group). This, and the observation that responses to bradykinin are potentiated by intradermal injection of PMN-leucocytes (Firrell, Peck & Williams, 1976), suggests that the source of the potentiating substance is the PMN-leucocyte.

Potentiation was suppressed by mixing the *B. pertussis* with indomethacin or dexamethasone before injection; (bradykinin = 35.1 ± 6.5 μl, *B. pertussis* = 13.1 ± 2.7 μl, *B. pertussis*/bradykinin = 95.3 = 6.8 μl, *B. pertussis* + indomethacin (1 μg/site)/bradykinin = 47.2 ± 4.2 μl, *B. pertussis* + dexamethasone (1 μg/site)/bradykinin = 50.0 ± 2.9 μl, $n = 6$ sites).

These results suggest that in response to the introduction of particulate materials into tissues, PMN-leucocytes infiltrate and then release a vasodilator, probably a prostaglandin, which is able to potentiate the exudation produced by other inflammatory mediators.

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## Salicylic acid prevents inhibition by aspirin of arachidonic acid-induced hypotension, bronchoconstriction and thrombocytopenia

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Bronchoconstriction, thrombocytopenia and hypotension by arachidonic acid (AA) are inhibited in aspirin-treated animals (Berry, 1966; Larsson & Änggård, 1973; Lefort & Vargaftig, 1975). Since salicylic acid and aspirin compete for albumin binding (Farr, 1971) and may antagonize each other (Ezer, Palosi, Hajos & Szporny, 1976), we investigated whether salicylic acid prevents inhibition by aspirin of the effects of AA. Pentobarbitone (30 mg/kg) anaesthetized guinea-pigs (i.p.) and rabbits (i.v.) were prepared for recording of blood pressure and, in case of guinea-pigs, of bronchoconstriction by the Konzett-Rössler method. Arterial blood was sampled for automatic platelet determinations and for *in vitro* aggregation (Born, 1962), using AA (0.01–0.1 mM) and ADP (0.01–1 μM). Platelet-rich plasma (PRP) prepared from citrated blood (3.8%, 0.1 vol), was incubated for 10–30 min with the drug solvent (polyethyleneglycol 300), with salicylic acid (1–4 mM) or with aspirin (0.05–0.1 mM), washed (Vargaftig, Tranier & Chignard, 1974), and resuspended in drug-free plasma or Tyrode solution. The aggregation behaviour was determined and assays for generation of thromboxane A2 activity on strips of superfused rabbit mesenteric artery and aorta carried out (Piper & Vane, 1969; Vargaftig & Dao, 1971; Bunting, Moncada & Vane, 1976). Hypotension in rabbits and bronchoconstriction and thrombocytopenia in guinea-pigs due to AA were unaffected by prior administration of salicylic acid (100–200 mg/kg i.v.) and were suppressed by aspirin (5 mg/kg i.v.). When salicylic acid was injected before aspirin at a 40 : 1 weight ratio, the latter failed to suppress the effects of AA. Platelet-rich plasma prepared from aspirin-treated animals was not aggregated by AA, and the ADP-induced second wave of aggregation in guinea-pigs was inhibited. Aggregation and generation of thromboxane A2 activity were obtained in AA-PRP incubates from animals pretreated with salicylic acid before aspirin. Incubation of PRP or of platelets resuspended in Tyrode solution containing bovine serum albumin or gelatin (0.35%) with aspirin, followed by resuspension in drug-free medium, resulted in suppression of aggregation and of generation of Thromboxane A2 due to AA and to bovine thrombin (2.5 μml$^{-1}$). Incubation with salicylic acid before adding aspirin prevented inhibition of the effects of AA and of thrombin. In contrast, incubation of platelets with amounts of warfarin (0.01–1 mM), expected to occupy most of the albumin binding sites (Koch-Weser & Sellers, 1976), failed to prevent aspirin-induced inhibition of platelet aggregation and generation of thromboxane $A_2$ due to AA or to thrombin. Salicylic acid antagonizes aspirin-induced inhibition of the *in vivo* effects of AA attributable to generation of prostaglandin intermediates and by-products. Since this antagonism occurs *in vitro* in absence of albumin, it does not result from competition for binding, and probably involves interaction at the cyclooxygenase level.

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# Selective inhibition of platelet responses to bisenoic prostaglandins

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Prostaglandins (PGs) and prostaglandin analogues exert a variety of effects on human platelets. $PGD_2$ and $PGE_1$ are potent inhibitors of aggregation (Ki = 20 nM), and $PGE_2$ is a weaker inhibitor (Ki = 20 μM) (MacIntyre & Gordon, 1975).

In contrast, methylated derivatives of $PGE_2$ and stable synthetic derivatives of $PGH_2$ are potent inducers of platelet aggregation, and appear to mimic the native prostaglandin endoperoxides (Gordon & MacIntyre, 1976; Corey, Gordon, MacIntyre & Salzman, 1977). In the present study, sodium-*p*-benzyl-4-(1-oxo-2-(4-chlorobenzyl)-3-phenylpropyl) phenyl phosphonate (N-0164), a prostaglandin antagonist and inhibitor of thromboxane synthase (Eakins, Rajadhyaksha & Schroer, 1976; Kulkarni & Eakins, 1976) was examined for its effect on human platelet responses to stimulatory and inhibitory prostaglandins.

Platelet aggregation and secretion of radio-labelled serotonin induced by arachidonic acid, 15(S)hydroxy-11α,9α-(epoxymethano) prostadienoic acid (U46619), 11-deoxy 15(S)-16(RS)-methyl-$PGE_2$ (Wy 19, 110) and ADP, were measured as described previously (Corey *et al.*, 1977). To investigate its inhibiting effect, N-0164 was preincubated in platelet-rich plasma (PRP) at 37°C before the addition of stimulatory prostaglandins (U46619 or Wy 19,110). In studies with inhibitory prostaglandins ($PGE_1$, $PGE_2$, $PGD_2$), N-0164 was preincubated in PRP for 1 min at 37°C before the addition of the prostaglandin, and 1 min later ADP was added, to induce platelet aggregation.

Both stimulatory prostaglandin analogues induced primary (reversible) aggregation at low concentrations (U46619 $\leqslant$ 0.2 μM; Wy 19,110 $\leqslant$ 0.3 μM) and secondary (irreversible) aggregation, accompanied by up to 60% release of radio-labelled serotonin, at higher concentrations. N-0164 (5–250 μM) caused a dose-dependent inhibition of aggregation and serotonin release induced by U46619 and Wy 19,110. Its inhibitory effect was not altered by preincubation, but was readily overcome by increasing the concentration of the agonist. In contrast, even after prolonged pre-incubation in PRP, N-0164 (250 μM) had little effect (<20% inhibition) on aggregation and serotonin release induced by arachidonic acid (0.5–2 mM), and had no effect on responses to ADP (0.5–3 μM). Inhibition by $PGD_2$ (60 nM) and $PGE_2$ (60 μM) of platelet aggregation induced by ADP (0.3–1 μM) was abolished by N-0164 (100–250 μM), but the inhibitory effect of $PGE_1$ (60 nM) was unaltered.

These results indicate that N-0164 antagonizes the effects of bisenoic prostaglandins. Inhibition of platelet aggregation by $PGE_1$, $PGE_2$, and $PGD_2$ is attributed to their stimulation of adenylate cyclase (for review, see Mills & Macfarlane, 1977); the failure of N-0164 to affect the action of $PGE_1$ while blocking $PGD_2$ and $PGE_2$ is the clearest demonstration so far that $PGE_1$ exerts its effect on a different receptor. Since arachidonic acid stimulates platelets through its endoperoxide and thromboxane metabolites, it is curious that N-0164 abolished the effect of endoperoxide analogues yet did not inhibit the response to arachidonic acid. However, the relative importance of these metabolites is not known, and since N-0164 does not inhibit the effects of thromboxane $A_2$ on rabbit aorta (Kulkarni & Eakins, 1976), the ineffectiveness of N-0164 against arachidonic acid could be because thromboxane $A_2$ is mainly responsible for arachidonic acid's effect on platelets. If so, this would imply that N-0164 does not inhibit thromboxane synthase in intact platelets. It is also possible that the arachidonic acid metabolites stimulate platelets via intracellular sites inaccessible to N-0164, and further work is necessary to characterize more fully the actions of this interesting compound.

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# Some characteristics of the metabolism of β-phenylethylamine in rat isolated lung

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β-Phenylethylamine (PEN) is metabolized on a single passage through the pulmonary circulation of rats and rabbits (Bakhle & Youdim, 1976; Roth & Gillis, 1975) and the metabolism of this amine involves monoamine oxidase of the B type (MAO-B; Yang & Neff, 1973) in contrast to that of 5-hydroxytryptamine (5-HT) which involves MAO-A. Both these enzymes are intracellular and amine metabolism must therefore be preceded by transfer of substrate across the cell membrane. Either step, uptake or enzymic reaction, could be rate-limiting.

We have studied the kinetics of the metabolism of PEN and of 5-HT in isolated rat lungs, by measuring the amine metabolites over a range of concentrations. Amine labelled with $^{14}C$ was infused through isolated lungs, perfused with Krebs solution via the pulmonary artery, for 3 min and the effluent collected during, and after, the infusion for a total of 30 minutes. By this time the radioactivity in the effluent had fallen to background levels. Aliquots of the collected effluent were chromatographed on ion exchange columns (Amberlite CG-50; Southgate & Collins, 1969) and the radioactivity eluted from the columns with water (non-basic metabolites) measured by liquid scintillation methods.

For PEN over a concentration range of 0.1–150 μM, metabolism seems to be governed by a single rate-limiting step with an apparent $K_m$ of 55 μM and $V_{max}$ of 880 nmol $lung^{-1}$ 30 $min^{-1}$. In contrast, the metabolism of 5-HT over a similar range (0.1–50 μM) seems best described by two processes; one with $K_m$ 2 μM and $V_{max}$ 34 nmol and the other with a $K_m$ value at least 25 times higher. The $K_m$ values for metabolism in the perfused organ should be compared with the $K_m$s for 5-HT, 187 μM, and PEN, 20 μM, obtained with MAO isolated from liver (Houslay & Tipton, 1974).

Metabolism of PEN in lung is not inhibited by 5-HT (Bakhle & Youdim, 1976) and in these experiments, tryptamine and benzylamine (0.2 and 2.0 μM) did not inhibit the metabolism of $^{14}C$-PEN (0.15 μM). Furthermore, the inactivation of noradrenaline (0.5–2 ng/ml) in isolated lungs, measured by bioassay, was not affected by PEN infused through the lung in concentrations up to 500 ng/ml. Tricyclic antidepressant drugs such as amitriptyline and imipramine inhibit uptake and hence metabolism of 5-HT and noradrenaline in lung (for refs. see Bakhle & Vane, 1974) but PEN metabolism was not inhibited by desmethylimipramine ($10^{-5}$ M). However, in lungs from rats pretreated (3 h; 5 mg/kg i.p.) with deprenil, an inhibitor of MAO-B (Knoll & Magyar, 1972), metabolism of PEN was inhibited by 34%.

From these results, the rat lung has a greater capacity to metabolize PEN compared with 5-HT and the process by which PEN enters the cell may be different from those uptake systems already described for 5-HT and noradrenaline in the lung (Gillis, 1976).

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# The metabolic fate of [$^{14}$C]-phenobarbitone in the rat and the effect of chronic administration and dose size

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The metabolic fate of phenobarbitone (5-ethyl-5-phenylbarbituric acid) has been studied in the rat as part of an examination of the biochemical basis of the tolerance which occurs to this drug. The effect of dose size and chronic administration on its metabolism has also been examined. [$^{14}$C]-Phenobarbitone, labelled in the 2-position of the barbituric acid ring (50 mg/kg; 10 μCi/animal), was injected intraperitoneally into female Wistar albino rats (body weight 180–200 g). Urine and faeces were collected for 8 days after administration and $^{14}CO_2$ in the expired air collected for 2 days. The excretion of $^{14}$C was monitored by liquid scintillation counting and the metabolites present in the urine examined by thin layer chromatography followed by radiochromatogram scanning, reverse isotope dilution, gas-liquid chromatography and gas chromatography-mass spectrometry (g.c.-m.s.). Over the 8 days of the experiment, some 88% of the administered $^{14}$C was recovered, with 79% in the urine, 0.14% as $^{14}CO_2$ in the expired air and 9% in the faeces. The bulk (75%) of the urinary $^{14}$C was excreted in the first 3 days, with 43% appearing in the first 24 hour. After the 4th day, some 0.5–1% of the dose was excreted in the urine per day. Analysis of the 0–24 h urine by the techniques described revealed the presence of unchanged phenobarbitone (14% of dose), *p*-hydroxyphenobarbitone, excreted free (16%) and as its glucuronide conjugate (9%) and an unknown acidic water-soluble metabolite (4%). The identities of these metabolites were confirmed by comparison of their g.c.-m.s. properties with standards, and the g.c.-m.s. of the unknown suggested that it was ethylphenylmalonylurea, arising from the ring opening of phenobarbitone. The nature of the faecal radioactivity was not determined. Phenobarbitone and its metabolites were excreted at similar rates, and since the excretion half life of total $^{14}$C was quite long (ca. 1.25 days) it would be expected that phenobarbitone and its metabolites would be accumulated when the drug was administered on a daily basis.

In other experiments, the excretion of [$^{14}$C]-phenobarbitone by bile-duct cannulated rats was examined. After the i.p. injection of 50 mg/kg (10 μCi/animal), 35% of the dose was excreted in the bile in 24 h with a further 18% in the urine. The excretion of $^{14}$C in the bile proceeded at a fairly constant rate of about 1.5% of dose/h over the 24 h of the experiment. The metabolites present in bile were examined as above, and comprised phenobarbitone (5% of dose) *p*-hydroxyphenobarbitone glucuronide (21%) and the unknown metabolite (9%). No free *p*-hydroxyphenobarbitone was found in bile.

The possible variation in the metabolism of phenobarbitone with dose was also studied. Using the methods described, rats were dosed with 5 mg/kg and 100 mg/kg [$^{14}$C]-phenobarbitone by i.p. injection (10 μCi/animal), and the excreta analysed for $^{14}$C and metabolites. With both these doses, the rate of excretion and nature of the metabolic products was essentially similar to the findings reported with a 50 mg/kg dose.

Analysis of the tissue distribution of $^{14}$C 24 h after the i.p. injection of [$^{14}$C]-phenobarbitone (50 mg/kg; 20 μCi/animal) showed that the bulk of the radioactivity remaining unexcreted was associated with the muscle, fat and bones of the carcass (16%) and in the blood (red cells 3%/ml; plasma 1%/ml). Some 7% of the dose was associated with the vital organs, in the order liver > kidney > lung > heart > gut wall > brain, the brain containing 0.2% of dose.

In further experiments, rats were treated daily with phenobarbitone (50 mg/kg i.p.) (treated group) or the injection vehicle, 90% propane-1,2-diol (2 ml/kg i.p.) (control group) for up to 14 days. Groups of rats were taken after 4 days or 14 days chronic administration, injected with [$^{14}$C]-phenobarbitone (50 mg/kg; 10 μCi/animal) and their urine and faeces collected for 3 days. Analysis of $^{14}$C and metabolites in the urine was performed as described. The excretion of $^{14}$C in the urine and faeces of both treated and control groups was the same as after the single 50 mg/kg dose, and the urine contained the same four metabolites. There were no marked differences between the groups, but there was a trend for the treated animals to excrete more of the acidic unknown metabolite and less free *p*-hydroxyphenobarbitone than after the acute administration.

It has long been known that phenobarbitone is a powerful inducer of the drug metabolizing enzymes of the liver microsomes (Remmer, 1962), and it is therefore interesting to see that repeated phenobarbitone administration does not markedly affect its rate of excretion or pattern of metabolism, although this is the case with many other compounds. In view of this, and of the possible accumulation of phenobarbitone and its metabolites in the body on chronic administration, it provides further support for the view that the tolerance which occurs to this drug does not have its origin in altered drug disposition (see Caldwell & Sever, 1974).

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# Self induction of metabolism by ketamine in the rat

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In a previous communication to the Society (Livingston & Waterman, 1976) we indicated that tolerance following repeated administration of ketamine was due to enzyme induction. We have now confirmed that pretreatment with ketamine shortens its duration of action and we have also investigated its effect on plasma levels of ketamine and its metabolites as well as on the rate of metabolism of ketamine by liver homogenate preparations *in vitro*.

The pretreatment schedule consisted of 10 daily injections of ketamine hydrochloride (40 mg/kg i.p.) the last injection being given 24 h prior to further experiment (control rats received saline injections). The duration of action of a single dose of ketamine (75 mg/kg i.p.) was then measured in control and treated rats. In addition, animals were killed at various times after injection in order to obtain blood for the assay of plasma levels of ketamine and its metabolites as previously described (Livingston & Waterman, 1976).

The pretreated rats, when compared to controls, showed a significant decrease in sleeping time (11.73 ± 0.95 compared to 23.11 ± 1.4 min), there was a significant decrease in their plasma levels of ketamine and metabolite I and a significant increase in their plasma metabolite II levels (Figure 1).

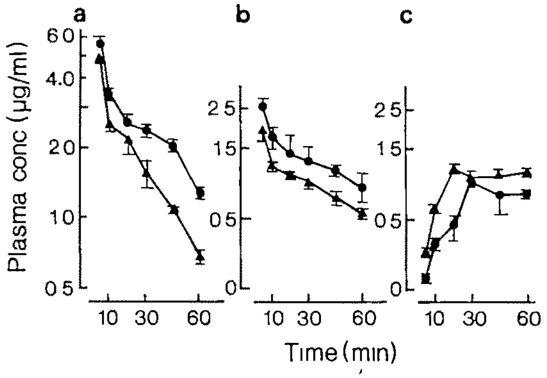

**Figure 1** Plasma levels of Ketamine (A) Metabolite I (B) and Metabolite II (C) after i.p. administration of Ketamine (75 kg/mg) to rats, pretreated with either Ketamine (▲) or saline (●). The rats were killed at the times indicated after injection and blood collected for assay of plasma levels of ketamine and its metabolites. Each point represents the mean of 4 animals ± s.e mean.

The metabolism of ketamine *in vitro* was investigated in homogenates of livers from control and ketamine pretreated rats using the method of Cohen & Trevor (1974). Samples (0.1 ml) were taken from the reaction vessels at zero time and after, 5, 10, 15, 30, 60 and 120 min incubation with ketamine (initial conc. 100 µg/ml) for the assay of ketamine and its metabolites.

The only detectable product of the metabolism of ketamine *in vitro* was metabolite I (the *n*-demethylated product) and pretreatment increased the rate of *n*-demethylation of the drug by an average of 122%. We have so far been unable to demonstrate the further metabolic conversion of metabolite I *in vitro*.

A.E.W. is a Wellcome Research Training Scholar.

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## On the detectability of anabolic steroids in horse urine

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The antibody raised against 19-nortestosterone 3-(*O*-carboxymethyl)oxime-bovine serum albumen in Soay ewes (Jondorf, 1977) has been characterized. It can be used for following the excretion of various anabolic steroid preparations administered to horses in veterinary doses.

Cross-bred and thoroughbred geldings maintained under standard conditions of feeding and exercise in our stables were injected intramuscularly with one of the following: 19-nortestosterone laurate (200 mg; 0.46 mg/kg), 19-nortestosterone phenylpropionate (400 mg; 0.75 mg/kg), 19-nortestosterone cyclohexylpropionate (100 mg; 0.25 mg/kg), 19-nortestosterone (150 mg; 0.40 mg/kg).

Urine samples collected from experimental animals before administration of anabolic steroid, and sequentially after administration, were kept deep-frozen (–20°C) until required for analysis. Urines were then thawed, adjusted to pH 9 and were extracted with equal volumes of chloroform/methanol (9 : 1 v/v) in rotary separators for 20 minutes. Extracts (1 ml) were analysed by radioimmunoassay (Jondorf, 1977) after removal of solvent, with [$^3$H]-19-nortestosterone as marker, and with unlabelled 19-nortestosterone as standards (100–2000 pg). For comparison, unextracted urine samples (0.01 ml) and residues after solvent extraction (0.01 ml) were also analysed in some instances.

The sensitivity of the radioimmunoassay method is such that a biological sample should contain more than 100 pg of 19-nortestosterone equivalent when analysed.

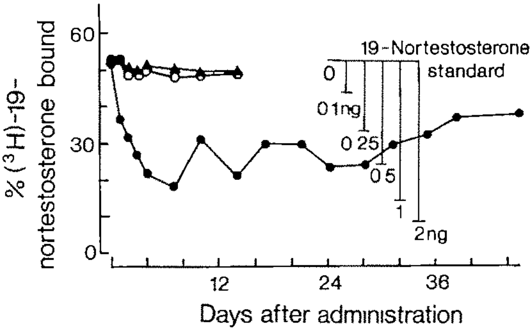

**Figure 1** The depression of binding of [$^3$H]-19-nortestosterone in horse urine after administration of 19-nortestosterone laurate (200 mg, i.m.). Radioimmunoassay with (●) 1 ml chloroform/methanol extract (O) 0.01 ml urine (▼) 0.01 ml urine after solvent extraction.

As shown in Figure 1, the urinary fate of 19-nortestosterone laurate can be followed for more than 45 days, at which time the depression of binding is still in the range equivalent to 100–250 pg 19-nortestosterone. It is known from studies on the metabolism of 19-nortestosterone in the rabbit (Both-Miedema *et al.*, 1972) and in the horse (Houghton, 1977) that the drug is metabolized to isomers of 5-oestrane-3,17-diols, and that these isomers cross-react with the antibody used in these assays (Jondorf, 1977). The detectability of anabolic steroid after administration of a veterinary preparation is not therefore dependent solely on the excretion of unchanged, de-esterified parent steroid.

In the corresponding experiments with the administration of other esters, the urinary detectability for the phenylpropionate and the cyclohexylpropionate were >15 days and >10 days respectively. 19-Nortestosterone itself was excreted fairly rapidly in contrast (detectable for ca. 2 days).

Among anabolic steroids cross-reacting with the antibody, and administered under the same conditions, trienbolone acetate (75 mg; 0.17 mg/kg) and 1-dehydrotestosterone undecylenate (250 mg; 0.66 mg/kg) could be detected >5 days and >21 days respectively.

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## Hypothalamic polypeptides and analogues on corticotrophin production by rat adenohypophysial tissue *in vitro*

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Corticotrophin releasing hormone (CRH), the hypothalamic releasing factor which controls the secretion of corticotrophin (ACTH) by the adenohypophysis, appears to be a polypeptide similar to but not identical with vasopressin. We have attempted to obtain more information about its precise nature by studying the corticotrophin releasing activity of hypothalamic polypeptides and their analogues. Their ability to stimulate corticotrophin synthesis and release by pituitary tissue *in vitro* was assessed. Segments of rat adenohypophysial tissue were incubated in medium containing the putative releasing hormone and the ACTH contents of the tissue and the incubation medium were then determined using a modification (Alaghband-Zadeh, Daly, Bitensky & Chayen, 1974) of the sensitive and precise cytochemical assay method (Chayen, Loveridge & Daly, 1972). Pressinoic acid, its amide, oxytocin, 8-alanine vasopressin, and the tail fragment (proline-arginine-glycinamide) of arginine vasopressin were ineffective and the desglycinamide derivatives of lysine- and arginine vasopressin were only slightly effective in stimulating ACTH synthesis and release. Arginine- and lysine vasopressin caused dose related increases in ACTH production but both were less active than oxypressin and arginine vasotocin. Arginine vasotocin was the most active compound studied and, in small doses which alone did not influence ACTH production, also markedly potentiated the action of hypothalamic extract.

The corticotrophin releasing activity of the polypeptides was also tested in pentobarbitone/chlorpromazine treated rats (de Wied, 1967) and, although the method is considerably less sensitive than the *in vitro* technique, the results were similar. Comparison of the corticotrophin releasing potencies of such compounds with hypothalamic extract, using different assay systems, may lead to a clearer understanding of the nature of the corticotrophin releasing hormone(s).

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# Effect of adrenaline on intracellularly recorded action potentials of mammalian skeletal muscle

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The most striking effect of adrenaline on isometric twitches of mammalian skeletal muscle is the altered time course of contraction; prolongation occurs in fast-twitch muscle and shortening in slow-twitch muscle. The rate of development of tension and the shortening velocity are not appreciably affected (Lewis & Webb, 1976). Such findings indicate an effect on $Ca^{++}$ release/re-uptake mechanisms which may operate by a change in the muscle action potential. Therefore, we have investigated the effect of adrenaline on intracellularly recorded action potentials (AP) in both fast-twitch flexor digitorum longus (FDL) and slow-twitch soleus muscle of the cat.

Experiments were performed on cats anaesthetized with pentobarbitone. Adrenaline (1 $\mu g\ kg^{-1}\ min^{-1}$ for soleus, 5 $\mu g\ kg^{-1}\ min^{-1}$ for FDL) was infused into the jugular vein and pressure in the common carotid artery was recorded in all experiments. The technique used for recording intracellularly with microelectrodes was similar to that described by Buller, Lewis & Ridge (1966) for mammalian muscle *in vivo*. Micropipettes were filled with 3 M–KCl and had resistances in the range 15–25 MΩ. Movement was limited by the use of a concentric needle electrode, ground flat at the tip, to stimulate a small bundle of fibres.

Control AP records from between 25–35 different fibres were obtained in the muscle under study and a similar number of fibres were sampled during the 40 min infusion period and again 1–1.5 h after the infusion. Results obtained in this way from 4 Soleus and 4 FDL muscles are given in Table 1.

Adrenaline had no significant effect on resting potential or measured amplitude and duration parameters of the action potential. We conclude its effect on activation processes in mammalian skeletal muscle is most likely on intracellular mechanisms, e.g. handling of $Ca^{++}$ by *t*-tubules or sarcoplasmic reticulum.

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**Table 1** Effect of adrenaline (ADR) on muscle action potential

| | *Flexor Digitorum Longus* | | | *Soleus* | | |
|---|---|---|---|---|---|---|
| | *Control* | *ADR (5 $\mu g\ kg^{-1}\ min^{-1}$)* | *Post-ADR control* | *Control* | *ADR (1 $\mu g\ kg^{-1}\ min^{-1}$)* | *Post-ADR control* |
| No. of fibres | 118 | 111 | 98 | 129 | 90 | 90 |
| Resting potential (mv) | 84.0 (0.79) | 82.0 (0.65) | 83.9 (0.81) | 82.9 (0.49) | 79.3 (0.83) | 77.1 (0.64) |
| Overshoot (mv) | 33.1 (0.86) | 33.9 (0.81) | 34.4 (0.95) | 20.2 (0.57) | 23.3 (0.95) | 21.7 (0.88) |
| Time above 0 mv (μs) | 382.7 (0.91) | 442.9 (0.61) | 422.5 (0.68) | 457.7 (0.51) | 476.5 (0.82) | 453.5 (0.80) |
| Time above −50 mv (μs) | 807.6 (1.69) | 866.3 (0.93) | 834.6 (1.23) | 1066.1 (0.95) | 1156.4 (1.75) | 1085.7 (1.24) |
| Time to peak (μs) | 382.1 (0.65) | 397.5 (0.55) | 375.4 (0.64) | 507.2 (0.79) | 464.9 (0.76) | 432.2 (1.18) |
| Rate of rise ($v.s.^{-1}$) | 1062.2 (18.5) | 912.8 (19.4) | 976.1 (22.8) | 630.1 (9.8) | 622.5 (16.5) | 719.1 (18.5) |

Figure in brackets are s.e. mean.

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## The effect of aspirin on the protein binding of ascorbic acid

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Tukamoto, Ozeki, Hattori & Ishida (1974) demonstrated the binding of ascorbic acid (AA) to bovine serum albumin (BSA) at 5°C and 20°C by the method of dynamic dialysis developed by Meyer & Guttmann (1968). Using this procedure, AA-BSA binding is shown to occur at 37°C. The presence of acetylsalicylic acid (ASA) is found to reduce this binding. The method of dynamic dialysis involves measurement of the rate of diffusion of AA from a dialysis sac in the presence and absence of BSA. The concentration of free and bound AA in the sac was calculated by measurement of AA diffusion rate. 100 ml aliquots of the Sorenson buffer, at pH 7.38, surrounding the sac were sampled and replenished with fresh buffer at 30 min intervals. AA was analysed by taking 0.5 ml of the sample and adding 4 ml of 0.2 M hydrochloric acid and reading the absorption on an ultra-violet spectrophotometer at 243mμ and compared with a standard curve. Initial sac concentrations of AA, BSA, and ASA were $2 \times 10^{-2}$ M, $2 \times 10^{-4}$ M, and $1 \times 10^{-2}$ M, respectively. All experiments were carried out under nitrogen to prevent the oxidative degradation of the AA. The molecular weight of BSA was taken as 69,000.

Typical results are normally shown in the form of a semi-log plot of total drug concentration against time. In the presence of AA alone, there is a rapid rate of diffusion. When BSA is also present, the rate of diffusion of AA is decreased over time. The slower rate of diffusion indicates that binding of AA with BSA is taking place within the dialysis sac. The degree of separation of these plots is an estimate of the extent of binding of AA to BSA.

The results of these experiments show that AA-BSA binding occurs at 37°C. The introduction of ASA into the sac containing AA and BSA causes the semi-log plot of total drug-concentration against time to approach the line obtained in the absence of BSA. This shows that ASA causes a reduction in the binding of AA to BSA.

By the method of Scatchard (1949) two types of binding sites were isolated for AA binding to BSA. There are ~6 sites per mole of protein of one type. The other sites are non-specific, low-affinity sites of lesser importance.

With ASA present the number of binding sites decreases to ~8 with no change being observed in the non-specific type.

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## Synaptosome transmitter release and ATPase activity

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It has been proposed that acetylcholine release at the periphery (Paton, Vizi & Zar, 1971) and in the central nervous system (Vizi, 1972) is triggered by inhibition of sodium, potassium-activated, magnesium-dependent adenosine triphosphatase ($Na^+$, $K^+$-ATPase). A stringent test of this hypothesis should include simultaneous measurements of acetylcholine release and ATPase activity. We have determined the effects of phenytoin, an inhibitor of nerve terminal $Na^+$, $K^+$-ATPase, and electrical stimulation on the release of acetylcholine and inorganic phosphate from synaptosomes during the same period to determine any relationship between the two.

Rat cerebral cortex synaptosomes were prepared as described previously (Gilbert & Wyllie, 1976). The synaptosomes were layered on filters and maintained at 37°C in an oxygenated medium (pH 7.4) containing (mM): NaCl (153.5); KCl (5.65); $MgSO_4.7H_2O$ (2.8); $CaCl_2.6H_2O$ (2.1); $NaHCO_3$ (1.8); glucose (8.3); sucrose (64.3); physostigmine (0.02). Phosphate and/or acetylcholine release were measured over 10 min periods with and without supramaximal electrical stimulation by platinum electrodes (100 Hz, 1 ms, 10 V). Phosphate was determined by the method of Bonting, Simon & Hawkins (1961) and acetylcholine by bioassay using the leech dorsal muscle.

Phenytoin, at a concentration which completely inhibits synaptosome $Na^+,K^+$-ATPase ($5 \times 10^{-5}$ M), decreased the basal release of phosphate. However, phenytoin did not alter basal acetylcholine release. When electrical pulses were applied acetylcholine release was increased considerably but phosphate release was also increased. Phenytoin prevented the evoked release of acetylcholine and it markedly reduced the evoked release of phosphate.

The question arises as to how much phosphate release is associated with $Na^+,K^+$-ATPase activity. Experiments involving a number of enzyme inhibitors suggested that approximately 25% of the basal release was limited to $Na^+,K^+$-ATPase while at least a further 65% resulted from the activities of other ATPases in the synaptosomes.

These results will be discussed in relation to the transmitter release-$Na^+,K^+$-ATPase hypothesis.

We thank G.D. Searle & Co. Ltd for financial support.

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## Effect of reserpine on choline acetyltransferase and high affinity choline uptake in the rat brain

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Regulation of the synthesis of acetylcholine (ACh) in response to nerve impulse flow has been proposed in central (Grewaal & Quastel, 1973) and peripheral (Collier & MacIntosh, 1969) tissues, and adaptational changes affecting neuronal ACh synthesis have been described. Thus choline acetyltransferase (ChA) activities are increased during neuronal activation by drugs (e.g. Mandell & Knapp, 1971; Oesch, 1974). Similarly, changes have been reported in the maximum rate of sodium-dependent high affinity choline uptake (ChU) into brain synaptosomes (Simon, Atweh & Kuhar, 1976). No information was available, however, about the relationship between adaptations of this kind. Reserpine had been shown to decrease ACh concentrations (Beani *et al.*, 1966) and to increase ChA activities in brain (Mandell & Knapp, 1971). We therefore sought adaptations in ChA in

**Table 1** Increase of striatal choline acetyltransferase (ChA) and choline uptake (ChU) after treatment of rats with reserpine

| *Treatment* | *ChA activity (nmol/min, 10 mg protein) (1)* | *% of control activity* | *Sodium-dependent uptake of $[^3H]$-choline (pmol/5 min, 10 mg tissue) (2)* | *% of control uptake* |
|---|---|---|---|---|
| Controls | 10.1 ± 0 29 | 100 | 3 74 ± 0 076 | 100 |
| Reserpine 14 h | 10.1 ± 0.31 | 100 | 4.09 ± 0.18* | 109* |
| 18 h | 11.9 ± 0.49 | 118* | 4.51 ± 0.11‡ | 121‡ |
| 20 h | 12.8 ± 0.86† | 127† | 4.79 ± 0.40‡ | 128‡ |
| 24 h | 10.6 ± 0.41 | 105 | 3.73 ± 0.41 | 100 |

All ChU values were corrected for sodium-independent (low affinity) uptake by subtracting blanks measured after incubations in a sodium-free medium. $[^3H]$-choline: 0.25 μM throughout

(1) Mean of duplicate determinations on 6 rats ± s.e. mean; control group, mean of 24 rats

(2) Mean of 4 determinations on 12 rats ± s.e. mean; control, 24 rats.

Student's 2-tail *t*-tests. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.002$

response to reserpine, and compared the time course of adaptations in both ChU and ChA.

Male Wistar rats (150–200 g) were injected with reserpine (7.5 mg/kg i.p.). The dose was repeated after 12 h and the rats were killed 14 to 24 h after the first injection. ChA in brain homogenates (10% w/v, 50 mM-sodium phosphate, pH 7.0, 0°C) was assayed radiometrically (Fonnum, 1975). The uptake of $[^3H]$-choline into prism shaped ($0.1 \times 0.1 \times$ approximately 0.5 mm) tissue slices, incubated in Krebs medium (5 min, 37°C) was measured.

Homogenates of corpus striatum from animals treated with reserpine for 20 h showed statistically significant increases in ChA compared with controls ($10.1 \pm 0.48$ to $12.8 \pm 0.86$ nmol/min, 10 mg protein, $P < 0.01$, $n = 12$). Activities in the cortex, midbrain, hypothalamus, cerebellum, and pons-medulla, however, did not show significant changes ($P > 0.05$). Striatal ChU was increased after 14 h and ChA after 18 h treatment. Increased values were found after 20 h and control values were re-established after 24 h treatment (Table 1).

Thus, the initial adaptation of striatal cholinergic neurones to reserpine involves an increase in ChU. There is a second phase, however, in which the increase in ChU is paralleled by an increase in ChA. This is consistent with the hypothesis that high affinity uptake and subsequent acetylation of choline are coupled in the rat (Barker & Mittag, 1975).

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## The effects of chloroquine and other retinotoxic drugs on axonal transport of proteins in rabbit vagus nerve

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Human and animal studies have demonstrated ocular damage as a result of administration of chloroquine and hydroxychloroquine (Nylander, 1967; Shearer & Dubois, 1967), thioridazine (Zinn, 1975), ethambutol (Roberts, 1974) and clioquinol (see Meade, 1975). A number of these drugs, notably thioridazine bear a chemical similarity to chlorpromazine. Since chlorpromazine inhibits the axonal transport of proteins in peripheral nerves through an action on microtubules (Edström, Hansson & Norström, 1973; Cann & Hinman, 1974) we have examined the ability of the above retinotoxic drugs to inhibit the axonal transport of labelled proteins in rabbit vagus nerves *in vitro*.

Vagus nerves with nodose ganglia attached were incubated in medium 199 for 24 h at 38.5°C in a series of two-compartment chambers (McLean, Frizell & Sjöstrand, 1975). Fifteen μCi of tritium-labelled leucine (L-(4,5-3H) leucine, 58 Ci/mmol) were added to the compartments containing the nodose ganglia and a ligature tied on the nerves 6 cm from the ganglia at the start of the experiment. Drugs are dissolved in medium 199 and added to either nerve or ganglion compartment. Tritium-labelled proteins, transported from the nerve cell bodies in the ganglia accumulated in the axons proximal to the ligature. The amount of TCA-insoluble radioactivity in 5 mm segments of each nerve was measured by liquid scintillation counting and expressed as a fraction of the TCA-insoluble radioactivity in the rest of the nerve. Ganglia were homogenized and the TCA-insoluble radioactivity expressed in relation to protein content.

A significant ($P < 0.05$) decrease in the accumulation of proteins in the 5 mm segment of nerve immediately proximal to the ligature was found after treatment of the nerve trunks with chloroquine and hydroxychloroquine at a concentration of $10^{-3}$ M and with thioridazine, clioquinol and chlorpromazine at $10^{-4}$ M. Ethambutol ($10^{-3}$ M) had no significant effect. The decrease in accumulation with chloroquine was greater than that with hydroxychloroquine at the same concentration. Both chlorpromazine and chloroquine at $10^{-3}$ M in the ganglion compartment caused a significant fall in protein synthesis.

The results indicate that, with the exception of ethambutol, the drugs have an inhibitory effect on the axonal transport of proteins *in vitro*. The extent to which this contributes to their retinotoxic action is unclear. Retinal RNA and protein biosynthesis are also affected by chloroquine *in vitro* at the same concentration (Giuffrida, Sjöstrand, Cambria, Serra, Vanella, Avitabile, Jarlstedt & Karlsson, 1975).

A study *in vivo* was also undertaken in which albino and pigmented rabbits were fed chloroquine diphosphate (100 mg/kg) three days per week for 6–8 months. Vagus nerves were then removed and the axonal transport of labelled proteins examined *in vitro* by the above method. No significant difference was found between control and drug-treated animals. These results with chloroquine are consistent with previous work which indicated a lack of effect of the drug on RNA and protein synthesis *in vivo* (Karlsson, Giuffrida, Jarlstedt, Serra & Sjöstrand, 1976).

Drugs were donated by: Bayer (chloroquine); Winthrop (hydroxychloroquine); Sandoz (thioridazine); Ciba-Geigy (clioquinol); Leo (chlorpromazine) and Lederle (ethambutol).

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# An examination of the action of tetrabenazine on peripheral noradrenergic neurones

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Tetrabenazine has been used recently in the treatment of various forms of chorea (McLellan, Chalmers & Johnson, 1974). Its action resembles that of reserpine, but it is less potent and shorter-acting (Quinn, Shore & Brodie, 1959). The present study has examined these differences, comparing the actions of tetrabenazine and reserpine on peripheral noradrenergic neurones of rats (male Wistar; weight range 200–220 g).

Tetrabenazine (100 mg/kg i.p.) markedly decreased catecholamine induced histofluorescence in the iris, mesenteric arterioles and hepatic portal vein. Depletion was maximal 4 h after injection and fluorescence intensity returned to normal 24 h after injection. Reserpine (0.5 mg/kg i.p.) produced a similar loss of histofluorescence from the same tissues but this effect was not maximal until 18 h after injection and histofluorescence was still sub-normal 4 days after injection.

Amine-depleted irides, venae cavae and mesenteries removed from rats ($n=5$ for each drug) either 4 h after tetrabenazine treatment (100 mg/kg) or 18 h after reserpine treatment (0.5 mg/kg), were incubated with noradrenaline ($5 \times 10^{-6}$ M) in Krebs' solution at 37°C for 15 minutes. After washing in Krebs' solution to remove unbound noradrenaline they were incubated with 2% glyoxylic acid (buffered at pH 7) and prepared for fluorescence microscopy. Tissues from tetrabenazine-treated rats contained noradrenergic nerve terminals which had been markedly repleted by incubation with noradrenaline. Those from the reserpine-treated rats showed no restitution of fluorescence after noradrenaline-treatment. Fluorescence was restored to reserpine-treated tissues if tranylcypromine ($3 \times 10^{-5}$ M) was included with noradrenaline in the incubation.

Ultrastructural studies supported these findings. Noradrenaline restored electron density to synaptic vesicles in nerve terminals of the iris from tetrabenazine-treated rats. Incubation with noradrenaline had no effect on the vesicles of terminals in reserpine-treated irides. Electron-density of vesicle cores in iris nerve terminals was also induced by incubation of tetrabenazine-treated tissues with 5-hydroxydopamine ($1 \times 10^{-4}$ M). This did not occur in reserpine-treated irides.

Hepatic portal veins were also removed from the treated rats and suspended between platinum-wire electrodes in Krebs' solution gassed with 95% $O_2$/5% $CO_2$ at 37°C. Log frequency/response curves to nerve stimulation (140 V, 200 μs, 10 s trains; 1–20 Hz) were recorded. The responses of veins, from tetrabenazine- or reserpine-treated rats to each frequency of stimulation were much lower than those obtained from veins of untreated animals. The veins were then incubated with noradrenaline ($5 \times 10^{-6}$ M) for 15 min and log frequency/response curves repeated. This treatment increased the slope of the curve for tetrabenazine-treated veins but had no effect on that obtained from reserpine-treated veins.

These findings are consistent with the suggestion that tetrabenazine causes a reversible impairment of vesicular uptake and storage of noradrenaline but that the effect of reserpine on a particular population of vesicles is irreversible.

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## The influence of pre-synaptic $\alpha$-adrenoceptors on the overflow of noradrenaline in the stimulated mouse vas deferens

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In sympathetically innervated tissues a negative feedback system regulates noradrenaline (NA) output via a pre-junctional $\alpha$-adrenoceptor (Langer, 1974). This receptor was implicated in the control of the twitch response of the mouse vas deferens (Marshall, Nasmyth, Nicholl & Shepperson, 1977). The present experiments provide direct evidence for the existence of this $\alpha$-adrenoceptor.

Eight vasa deferentia were tied together and stimulated at 256 mA, 1 ms for 120 pulses at 1, 10 or 16 Hz. Contractions were recorded isometrically. The bath was emptied 3.5 min (or 5 min when drugs were present) after stimulation and the Krebs solution was assayed for NA by the specific radio-enzymatic method of Henry, Starman, Johnson & Williams, 1975.

Although the tension developed by the vasa was greater with increasing stimulation frequency, NA overflow did not increase proportionately. Addition of cocaine (10 μM) and oestradiol (3.7 μM)to the Krebs significantly inhibited the twitch (52.7%) at 1 Hz ($P < 0.05$) but not at 10 Hz or 16 Hz. However, at all three rates of stimulation NA overflow was approximately doubled. When phentolamine (10 μM) was added to the Krebs containing cocaine and oestradiol, the inhibition seen at 1 Hz was reversed and NA overflow was now increased a further 5-fold, a 12-fold increase over controls.

These results show that inhibition of the twitch response by cocaine and oestradiol (Marshall, Nasmyth & Shepperson, 1977) at 1.0 Hz correlates with increased NA overflow. In other tissues regulation of NA output is mediated by pre-junctional $\alpha$-adrenoceptors only at low rates of stimulation (Starke, Endo & Taube, 1975). In agreement with this, phentolamine produced a much greater increase in NA overflow at low rates of stimulation.

In other experiments vasa deferentia were pre-incubated for 45 min with 100 ng/ml of [7-$^3$H]-(−)-noradrenaline (specific activity 5.8 Ci/mmol). The [$^3$H]-catechol overflow from 4 vasa after 120 stimuli at 0.2, 1.0 or 10 Hz, 2 ms, 256 mA was absorbed onto alumina and counted.

Results were similar to those obtained by measuring NA overflow. However, as 0.2 Hz was used, the rate-dependent increase in [$^3$H]-catechols in the presence of cocaine (10 µM), oestradiol (3.7 µM) and phentolamine (10 µM) was more clearly seen.

The selective pre-junctional $\alpha$-adrenoceptor agonist clonidine (11.2 nM) inhibited the twitch by 68.3% at 0.2 Hz, by 24.0% at 1.0 Hz and 0.3% at 10 Hz. Clonidine (11.2 nM) reduced the overflow of [$^3$H]-catechols at 0.2 Hz but not at 1.0 Hz or 10 Hz.

The results, obtained from the measurement of NA overflow and the effect of clonidine, demonstrate the presence of a pre-synaptic $\alpha$-adrenoceptor in the mouse vas deferens.

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## Release of [$^3$H]-noradrenaline by field stimulation and by drugs from the anococcygeus muscle

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Incubation of rat anococcygeus with [$^3$H]-noradrenaline ($^3$H-NA) results in accumulation of the [$^3$H]-NA in the adrenergic nerve terminals (Nash, Gillespie & Robertson, 1974). This preparation is thus of potential use for the study of mechanisms involved in the release of NA.

Single rat anococcygeus muscles were incubated in a 3 ml bath under isometric conditions at an initial resting tension of 0.5 g in Kreb's solution at 37°C with [$^3$H]-NA (NA, 0.5 µM; 5 µCi/ml h; 1-(7-$^3$H) NA acetate, Radiochemical Centre, Amersham) for 30 min (EDTA 1.3 µg/ml; ascorbic acid 20 µg/ml also present). The tissues were then held isometrically in air under an initial resting tension of 0.5 g and superfused with drug-free Krebs' solution at 37°C at a rate of 0.66 ml/min. Sequential 2 ml aliquots of superfusate (corresponding to 3 min periods) were collected, 1 ml of which was mixed with 10 ml Toluene-Triton X scintillation fluid and the radioactivity counted in a liquid scintillation counter (Packard Tri-Carb, Model 3390) for 5 minutes. The tissue concentration of $^3$H measured at the end of several experiments divided by the $^3$H content of the corresponding incubation medium gave a ratio of $4.5 \pm 0.5$ ($n=6$). In a further three experiments where the tissue was digested immediately after incubation with [$^3$H]-NA, mean tissue/medium ratio was $5.5 \pm 1.3$ ($n=3$). Isometric tension in the tissue was measured throughout.

Following incubation with [$^3$H]-NA the quantity of $^3$H in the superfusate from the anococcygeus muscles showed an exponential decay with time which could be separated into two log-linear components with half-lives respectively of $25 \pm 5$ and $116 \pm 10$ min ($n=6$). Test drugs were therefore added 75 min after starting perfusion when the decay was essentially log-linear.

LSD (5 µM) or tyramine (5 µM) increased this spontaneous overflow whereas barium chloride (8 mg/ml) or carbachol (3 µM) did not although each substance contracted the tissue to a similar degree.

Field stimulation of the tissue with 1 ms pulses, supramaximal voltage, 150–300 pulses, at 5–20 Hz produced reproducible increases in the basal efflux of $^3$H in the 6 min period following stimulation. LSD (5 µM) inhibited both this latter nerve induced efflux and the accompanying motor tension response.

This confirms, as previously shown indirectly in this tissue, (Gillespie & McGrath, 1975), that LSD possesses the dual properties of indirect sympathomimetic and pre-synaptic inhibitor of NA release from adrenergic nerve terminals and illustrates that the preparation can be used to study the release of NA by nerve stimulation or by drugs.

Preliminary experiments indicate that nerve-induced $^3$H overflow can be potentiated by blockade of neuronal NA uptake (cocaine 1 µM) or by a pre-synaptic $\alpha$-receptor antagonist (piperoxan 3–30 µM).

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## Monoamine oxidation in tissues of the developing chick

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Monoamine oxidase (MAO) activity in several animal tissues is related to growth (see Gripois, 1975). For example, in the rat heart, its specific activity increases with age due to a slowing of the rate of degradation of the enzyme (Callingham & Della Corte, 1972). Changes in the thyroid status modify this relationship by changing the rate of synthesis (Lyles & Callingham, 1974). The domestic fowl, which, at hatching is an independent but immature animal, has been used in this present study.

The hearts and livers of male chicks were homogenized in 1 mM potassium phosphate buffer, pH 7.4. MAO activity was assayed radiochemically using [$^{3}$H]-tyramine and [$^{14}$C]-benzylamine, and expressed as nmol substrate consumed (mg protein)$^{-1}$ h$^{-1}$. MAO specific activities were determined in the hearts and livers of groups of 4 chicks, at 6, 21, and 40 days after hatching. In both organs there was an increase in enzyme activity with age using either substrate.

Chicks were made hypothyroid by the addition of 0.2% (w/w) of 2-thiouracil in the feed, beginning the day after hatching. At 40 days after hatching, the treated chicks were about 70% of the control weight, and their hearts 60%. In the liver there were significant increases in MAO specific activity, 2.4 and 2.1 times control for tyramine and benzylamine respectively. In the heart there was a significant decrease in activity towards benzylamine (56% of control) but no significant change in tyramine oxidizing activity.

Chicks were made hyperthyroid by daily injections of (−)-thyroxine (1 mg/kg, s.c.) for 11 days, beginning on the second day after hatching, and killed 24 h after the last injection. At this time, the hyperthyroid birds had an increased temperature, a body weight 80% of that of the controls, but no change in heart weight. In the heart there were significant increases in MAO specific activity to 1.4 and 1.5 times control for tyramine and benzylamine respectively. No significant changes in activity were seen in the liver.

Experiments using clorgyline showed that tyramine oxidation in the heart was brought about by MAO-A and -B together with a clorgyline-resistant enzyme. Benzylamine oxidation in the heart was brought about wholly by this clorgyline-resistant enzyme, which was, however, inhibited by semicarbazide. In the liver, MAO-A and -B were responsible for all the tyramine oxidation, but MAO-B and the clorgyline-resistant enzyme oxidized benzylamine.

These results indicate that the enzymes responsible for the oxidation of tyramine and benzylamine in chick heart and liver can be resolved into MAO-A and -B (Johnston, 1968) and a clorgyline-resistant enzyme (Lyles & Callingham, 1975) that does not appear to be flavine-dependent. The activities of these enzymes are influenced by the development and thyroid status of the bird.

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# Role of tryptamine in the behavioural changes caused by a monoamine oxidase inhibitor and L-tryptophan

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Giving L-tryptophan (L-TP) to rats pretreated with the monoamine oxidase (MAO) inhibitor tranylcypromine causes behavioural changes including hyperactivity, reciprocal forepaw treading, head weaving, Straub tail and hind limb abduction which depend on 5-hydroxytryptamine (5-HT) synthesis as they are prevented by tryptophan hydroxylase inhibition (Grahame-Smith, 1971). As brain tryptamine also increases when L-TP is given after MAO inhibition (Saavedra & Axelrod, 1973; Marsden & Curzon, 1974) it could also be involved and its role was therefore investigated.

In experiment 1 (Table 1) the increase of locomotor activity after tranylcypromine was greatly enhanced by L-TP, 50 mg/kg (+94% v. tranylcypromine alone). Addition of tryptamine (0.75 mg/kg) increased this further (+30% v. tranylcypromine + L-TP) but increased the behaviour score more strikingly (+76%). Brain 5-HT and tryptamine rose after L-TP (+56%, +64% respectively v. tranylcypromine alone) and tryptamine rose further when it was also given (+128% v. tranylcypromine + L-TP). These results suggest that the tryptamine changes after giving tranylcypromine + L-TP are sufficient to influence behaviour.

This is also supported by experiment 2 (Table 1) in which increasing the L-TP dose to 100 mg/kg increased locomotor activity slightly (+21% v. tranylcypromine + L-TP, 50 mg/kg) but the behavioural score considerably (+159%) in association with slight (+23%) and considerable (+107%) rises of brain 5-HT and tryptamine respectively.

Therefore, increased brain tryptamine on given L-TP after tranylcypromine may alter behaviour. However, this effect of tryptamine requires 5-HT as in confirmation of Foldes and Costa (1975) we find the behavioural effects of tranylcypromine (20 mg/kg) + tryptamine (1 mg/kg) are partly prevented by the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (3 × 100 mg/kg).

**Table 1** Behavioural and biochemical effects of L-tryptophan (L-TP) and tryptamine in tranylcypromine (TCP) treated rats

| | *Behaviour* | | *Biochemical determinations* | | |
|---|---|---|---|---|---|
| | | | | *5-HT* | |
| | | | *Brain* | | |
| | | | *(µg/g wet wt.)* | | |
| *Treatment* | *Locomotor activity* | *Behaviour score* | *L-TP* | | *Tryptamine* |
| *Exp. 1* | | | | | |
| Control (0.9% saline) | 1224 ± 329(4) | ND | 2.3 ± 0.5(9) | 0 64 ± 0.08(9) | ND |
| TCP (20 mg/kg) | 3765 ± 917(7)‡ | ND | 2.5 ± 0.3(15) | 0.97 ± 0.14(15)‡ | 0.11 ± 0.03(6) |
| TCP (20 mg/kg) + L-TP (50 mg/kg) | 7279 ± 433(8)‡ | 13.0 ± 1.8(8) | 17.7 ± 2.8(18)‡ | 1.51 ± 0.23(18)‡ | 0 18 ± 0.05(6)* |
| TCP (20 mg/kg) + L-TP (50 mg/kg) + Tryptamine (0.75 mg/kg) | 9507 ± [illegible] 4(4)‡ | 35.9 ± 3.4(4)‡ | 16.4 ± 2.4(12) | 1.51 ± 0.16(12) | 0.41 ± 0.06(6)‡ |
| *Exp. 2* | | | | | |
| TCP (20 mg/kg) + L-TP (50 mg/kg) | 6434 ± 510(4) | 12.3 ± 2.4(4) | 23.8 ± 5 4(10) | 1.26 ± 0.15(10) | 0.15 ± 0.05(5) |
| TCP (20 mg/kg) + L-TP (100 mg/kg) | 7767 ± 576(4)* | 31.8 ± 1.3(4)‡ | 45.9 ± 4.8(12)‡ | 1.56 ± 0.17(12)† | 0.31 ± 0.08(6)† |

All injections were i.p. Tranylcypromine was injected 30 min before other drugs and rats killed 60 min after the second injection. Locomotor activity is total counts (Animex) during these 60 min. Behaviour was scored (head weaving, forepaw treading, hind limb abduction, Straub tail) every 15 min during the 60 min (the total score is given). Tryptophan and 5-HT determined in single brains (Marsden & Curzon, 1974) and tryptamine on bulked extracts of two brains (Sloan, Martin, Clements, Buchwald & Bridges, 1975). Figures in brackets are number of values obtained; each behavioural value is on a cage of three rats Values given as mean ± s.d. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ all with respect to corresponding value on preceding line. ND = not done.

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# Monoamine oxidase activity at different levels of rat spinal cord

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Monoamine oxidase (MAO) exists in at least two forms, 'A' and 'B' which exhibit different substrate affinities and inhibitor susceptibilities (Squires, 1972). Rat spinal cord MAO exhibits 80% type A activity and 20% type B activity (George & Jones, 1976). In this study, MAO activity was investigated in different regions of rat spinal cord. Male rats 200–250 g were anaesthetized and the spinal cord was divided, *in situ*, into its cervical, lumbar, thoracic and sacral segments. The MAO activity of homogenates of each cord region was determined as previously described by George & Jones (1976). Each homogenate sample was incubated with [$^{14}$C]-tyramine (0.3 μCi) which is a substrate for both MAO types. The protein concentration of each homogenate sample was determined as described by Lowry, Roseborough, Farr & Randall (1951) and the MAO activity was expressed as ng product mg protein$^{-1}$ h$^{-1}$. By separate assays, the percentage inhibition of MAO produced by clorgyline ($1 \times 10^{-7}$ M) a specific type A inhibitor and pargyline ($1 \times 10^{-7}$ M) a specific type B inhibitor was determined for homogenates of each spinal region.

**Table 1** Differential MAO inhibition in spinal cord

| | *% Inhibition of MAO activity* | | *Ratio type A/B MAO activity* |
|---|---|---|---|
| *Tissue* | *Clorgyline ($1 \times 10^{-7}$ M)* | *Pargyline ($1 \times 10^{-7}$ M)* | |
| Whole cord | 81.9% | 23.2% | 4:1 |
| Cervical cord | 70.7% | 28.9% | 2.3:1 |
| Thoracic cord | 90.7% | 9.8% | 9:1 |
| Lumbar cord | 61.3% | 39.4% | 1.5:1 |
| Sacral cord | 82.5% | 21.2% | 4:1 |

George & Jones (1976) have demonstrated that the ratio of type A/B MAO in rat brain is 1.5:1. The present results show that for whole spinal cord the ratio was 4:1 while for the thoracic section it was 9:1. A ratio of 9:1 has been demonstrated in sympathetic ganglia (Goridis & Neff, 1971).

It is suggested that differences in MAO ratios between regions of the spinal cord may be related to the distribution of efferent pathways and neurotransmitters in the cord.

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# Modification by ethinyloestradiol and progesterone of the effects of imipramine on 5-hydroxytryptamine metabolism in discrete areas of rat brain

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Fludder & Tonge (1975) have shown changes in monoamine metabolism in eight areas of rat brain during the oestrous cycle which may be expected to modify both behaviour and the actions of psychotropic drugs. The fluctuations in the relative levels of endogenous oestrogen and progesterone which occur during female reproductive cycles may be directly responsible for the changes in brain monoamine concentrations at these times (Greengrass & Tonge, 1974).

The effects of imipramine, alone and in combination with ethinyloestradiol or progesterone, have been examined in female rats ovariectomized six weeks prior to use and in litter-mate intact females. Ethinyloestradiol and progesterone administration have been found to modify the effects of imipramine on 5-hydroxytryptamine (5-HT) metabolism in the eight areas of brain described by Fludder & Tonge (1975); the effects in two of these areas, the amygdala (representing brain areas containing a high proportion of neurone terminals) and the midbrain (representing areas containing principally axons/cell bodies), are shown in Table 1.

5-HT depletion after synthesis blockade with *p*-chlorophenylaline (PCPA; 400 mg/kg) was slower in the midbrain and faster in the amygdala in ovariectomized than in dioestrous rats, suggesting reduced and accelerated turnover rates respectively in the two areas. Depletion was accelerated by ethinyloestradiol and reduced by progesterone (unpublished results).

Imipramine failed to cause a statistically significant change in indoleamine concentrations in ovariectomized rats. However, when administered together with either ethinyloestradiol or progesterone, the same dose of imipramine produced effects similar to those seen in intact dioestrous rats. It therefore appears that the detectable effects of imipramine upon central 5-HT metabolism are dependent on the existent levels of oestrogen and/or progesterone and it is conceivable that the actions of the drug may be similarly susceptible in the human female.

It is suggested that both progesterone and imipramine reduce the uptake of 5-HT into nerve terminals, thereby increasing the concentrations of the amine at the receptor sites Ethinyloestradiol has been shown to affect both the turnover and the synthesis (unpublished results) of 5-HT and these effects may explain the influence of the hormone upon the actions of imipramine.

**Table 1** Effects of ethinyloestradiol and progesterone on the changes in 5-HT and 5-HIAA concentrations produced by imipramine in two areas of rat brain

| | *5-HT/5-HIAA (n moles/g ± s.e. mean)* | *Amygdala* | *Midbrain* |
|---|---|---|---|
| Intact rats at dioestrus (D) | 5-HT | 4.35 ± 0.14 | 2.98 ± 0.14 |
| | 5-HIAA | 0.97 ± 0.02 | 3.35 ± 0.01 |
| D + imipramine (cf. D) | 5-HT | 7.34 ± 0.05‡ | 4.40 ± 0.09‡ |
| | 5-HIAA | 0.71 ± 0.05‡ | 1.46 ± 0.06‡ |
| Ovariectomized litter-mates (O) | 5-HT | 4.18 ± 0.07 | 1.45 ± 0.04 |
| | 5-HIAA | 0.95 ± 0.09 | 5.24 ± 0.45 |
| O + imipramine (cf. O) | 5-HT | 4.73 ± 0.17 | 1.11 ± 0.08* |
| | 5-HIAA | 1.10 ± 0.06 | 4.14 ± 0.09 |
| O + EO | 5-HT | 3.35 ± 0.19 | 1.05 ± 0.20 |
| | 5-HIAA | 0.76 ± 0.04 | 4.14 ± 0.32 |
| O + EO + imipramine (cf. O + EO) | 5-HT | 4.73 ± 0.25† | 2.70 ± 0.23‡ |
| | 5-HIAA | 0.70 ± 0.03 | 3.89 ± 0.11 |
| O + progesterone | 5-HT | 2.88 ± 0.26 | 1.80 ± 0.07 |
| | 5-HIAA | 0.66 ± 0.04 | 2.79 ± 0.10 |
| O + progesterone + imipramine (cf. O + progesterone) | 5-HT | 3.30 ± 0.08 | 2.47 ± 0.08‡ |
| | 5-HIAA | 0.63 ± 0.01 | 2.85 ± 0.11 |

Statistically significant differences –Student's *t* test) are shown as * $P<0.05$, † $P<0.01$, ‡ $P<0.001$ Each value is the mean of determinations on 5 brains.

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# Effects of testosterone and ethinyloestradiol on the synthesis and uptake of noradrenaline and 5-hydroxytryptamine in rat hindbrain: evidence for a presynaptic regulation of monoamine synthesis?

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The participation of brain monoamines in the regulation of pituitary secretory activity is well established (Ganong, 1975) and the feedback control of plasma levels of gonadal steroids is dependent upon the ability of oestrogens, progestogens and androgens to affect central catecholamine and 5-hydroxytryptamine (5-HT) metabolism. Some of the actions of injected hormones on monoamine metabolism are presumably identical to those of endogenous hormones, with the caveat that there may be additional dose-dependent effects. Studies of the effects of hormones on defined neuronal processes may, therefore, provide some information on the physiological regulation of monoaminergic function as opposed to the pharmacological changes produced by foreign drugs.

The effects of testosterone (10 mg/kg s.c.) and

**Table 1** Effects of testosterone (T) and ethinyloestradiol (EO) on the uptake and synthesis of noradrenaline (NA) and 5-HT in two hind-brain regions of male rats

| | *5-HT (n moles/g ± s.e. mean)* | | *NA (n moles/g ± s.e. mean)* | |
|---|---|---|---|---|
| *Treatment* | *Midbrain* | *Pons/medulla* | *Midbrain* | *Pons/medulla* |
| None | 4.48 ± 0.17 | 3.68 ± 0.07 | 7.30 ± 0.48 | 4.94 ± 0.44 |
| Testosterone (T) | 3.59 ± 0.19 | 3.24 ± 0.23 | 6.49 ± 0.33 | 4.54 ± 0.31 |
| Phenelzine (P) | 4.79 ± 0.18 | 4.24 ± 0.27 | 8.90 ± 0.52 | 6.23 ± 0.25 |
| P + T | 8.16 ± 0.09 | 6.85 ± 0.29 | 11.07 ± 0.95 | 6.66 ± 0.60 |
| % age change due to T | +120% | +96% | +49% | +20% |
| None | 5.64 ± 0.41 | 5.23 ± 0.19 | 6.11 ± 0.44 | 4.07 ± 0.13 |
| Ethinyloestradiol (EO) | 5.49 ± 0.23 | 5.91 ± 0.26 | 5.67 ± 0.22 | 4.72 ± 0.30 |
| Phenelzine (P) | 6.87 ± 0.38 | 5.83 ± 0.14 | 6.80 ± 0.18 | 5.12 ± 0.22 |
| P + EO | 8.12 ± 0.47 | 7.48 ± 0.46 | 7.04 ± 0.34 | 5.28 ± 0.25 |
| % age change due to EO | +26% | +15% | +13% | +14% |
| None | 8.94 ± 0.80 | 6.22 ± 0.33 | 2.82 ± 0.13 | 2.64 ± 0.20 |
| Testosterone (T) | 6.18 ± 0.38 | 4.59 ± 0.27 | 2.41 ± 0.27 | 3.19 ± 0.42 |
| H75/12 | 6.18 ± 0.42 | 4.24 ± 0.16 | | |
| H77/77 | | | 2.06 ± 0.40 | 2.35 ± 0.14 |
| T + H75/12 or H77/77 | 6.18 ± 0.42 | 3.88 ± 0.07 | 3.90 ± 0.39 | 2.45 ± 0.28 |
| % age change | −31% | −16% | −14% | 0 |
| None | 6.76 ± 0.60 | 4.74 ± 0.40 | 5.03 ± 0.24 | 3.83 ± 0.36 |
| Ethinyloestradiol (EO) | 5.53 ± 0.20 | 3.60 ± 0.20 | 5.75 ± 0.33 | 4.12 ± 0.16 |
| H75/12 | 6.07 ± 0.17 | 3.95 ± 0.17 | | |
| H77/77 | | | 4.88 ± 0.33 | 3.77 ± 0.21 |
| EO + H75/12 or H77/77 | 5.79 ± 0.29 | 4.03 ± 0.11 | 5.39 ± 0.20 | 3.83 ± 0.29 |
| % age change | −15% | −24% | 0 | 0 |

Each value is the mean of determinations from 5 rats The % age changes shown are statistically significant (Student's *t* test) at least the $P < 0.05$ level.

ethinyloestradiol (100 μg/kg s.c.) on the rates of depletion of noradrenaline and 5-HT following 4-α-dimethyl-*m*-tyramine (H77/77) or 4-methyl-α-ethyl-*m*-tyramine (H75/12) respectively (2 x 12.5 mg/kg) have been determined as a measure of the effects of the hormones on the reuptake of monoamines by the neuronal pumps (Carlsson, Corrodi, Fuxe & Hokfelt, 1969a, b). An indication of the effects of the hormones on monoamine synthesis has been obtained by examining the accumulation of noradrenaline and 5-HT 4 h after 100 mg/kg (i.p.) phenelzine (Neff & Costa, 1968). The results of these determinations are shown in Table 1. The dissection and analytical techniques followed the method of Fludder and Tonge (1975).

Carlsson *et al.* (1969a, b) suggest that the depletions caused by the substituted *m*-tyramines are due to the displacement of monoamines because of the high affinity for the concentrating mechanisms. The data presented in Table 1 therefore suggest that both testosterone and ethinyloestradiol cause decreases in the uptake of noradrenaline and 5-HT and may thereby increase effective concentrations in the two brain areas shown here. The fact that testosterone and ethinyloestradiol have similar effects is consonant with the proposition that androgens may be aromatized to oestrogenic compounds in the brain before exerting some of their actions (Naftolin, Ryan & Petro, 1972).

It is apparent from the results presented in Table 1 that decreased amine uptake is accompanied by increased amine synthesis, suggesting that either steroid effects on the two processes are unrelated, or that synthesis may be regulated by presynaptic intraneuronal levels of free noradrenaline and 5-HT.

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# DEMONSTRATIONS

## Studies of the actions of drugs affecting the central nervous system and of the relationship between nerve terminal ATPase activities and neurotransmitter release

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A centrally-acting drug may mediate its action by altering neurotransmitter release. This effect could, in turn, result from inhibition or stimulation of enzymes in nerve terminals of the central nervous system.

The demonstration shows techniques which may be used to prepare nerve terminals (synaptosomes) from rat brain (homogenization, differential and density gradient centrifugation), a technique to measure the release of neurotransmitters from these terminals in the presence and absence of drugs *in vitro,* and the results of experiments to determine the effects of the same drugs on the activities of enzymes in the terminals. The enzymes principally studied here are ATP-hydrolyzing enzymes (ATPases) which are activated to different extents by sodium, potassium and magnesium ions. When synaptosomes are burst by osmotic shock it is possible to separate the mitochondria, membranes and vesicles from them and to study the effects of the drugs on the enzymes in these fractions independently. Some results are shown. A number of centrally-acting drugs inhibit ATPases in different components of the synaptosome but of the drugs tested so far only those with anticonvulsant activity inhibit the magnesium-ATPase located in the vesicles.

The effects of the drugs on the enzymes can be compared with their effects on the basal and evoked release of neurotransmitters from intact synaptosomes maintained *in vitro.* This comparison may throw light on the roles of the enzymes in neurotransmitter uptake and release processes.

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## On-line computation of peripheral resistance and stroke volume in the conscious cat obtained by the use of a chronically-implanted electromagnetic flow transducer

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The use of electromagnetic flow transducers to determine cardiovascular parameters in conscious dogs has not proved totally successful in the chronic situation because of the occurrence of aortic rupture (Stone & Sawyer, 1966; Folts & Rowe, 1973). We therefore decided to investigate the use of the cat as an alternative species since the cat has been shown to be particularly useful for cardiovascular measurements in the conscious state (Poyser, Shorter & Whiting, 1974). Aortic blood flow in the conscious cat was determined using an electromagnetic flow transducer placed around the ascending aorta. In addition, arterial blood pressure was measured using an indwelling aortic cannula (Day & Whiting, 1972).

The interpretation of drug-induced changes of the cardiovascular system is improved if determinations of peripheral resistance and stroke volume are made in addition to those of blood pressure and aortic flow. The assessment of these changes over periods of up to 8 h can be greatly simplified by using the on-line computer shown, in block diagram form, in Figure 1.

With this circuit the inputs, arterial blood pressure, aortic blood flow and heart rate, are first averaged using filters with a time constant of 15 seconds. This time constant was chosen as it provides an accurate representation of the effects upon peripheral resistance and stroke volume of both short and long-acting compounds whilst removing beat-to-beat variations. Subsequent to this averaging, mean arterial blood pressure is divided by mean aortic flow (this quantity is taken to be a reasonable approximation to cardiac output neglecting the 5% or so loss via the coronary arteries) to yield peripheral resistance, and mean aortic flow is divided by mean heart rate to give stroke volume. The units of peripheral resistance are presented in kPa $l^{-1}$ minute. This use of mixed SI and traditional units has been recommended (Kappagoda & Linden, 1976) in situations where changes in flow rate are mainly consequent upon

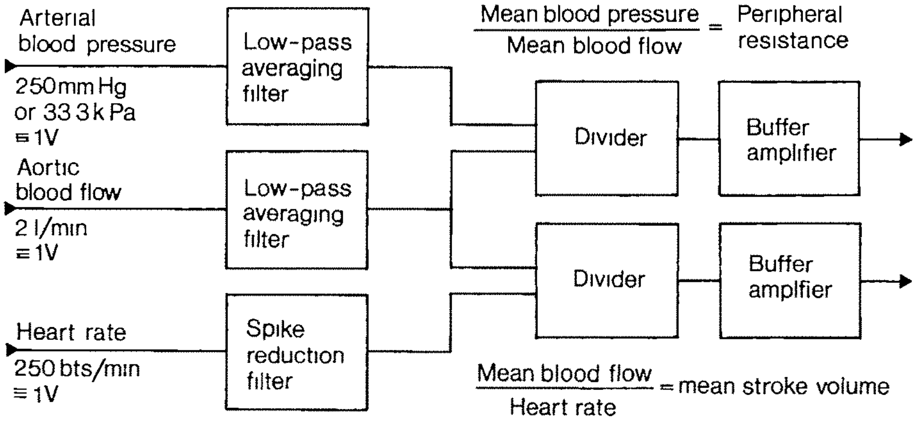

**Figure 1**

changes in vessel bore. The derivation of stroke volume used in this system eliminates the need for re-set integrators used by previous workers (Pinardi, Sainz & Santiago, 1975).

Preliminary tests with hydrallazine, clonidine, methyldopa, bufuralol, propranolol and some anti-hypertensive compounds synthesized within our laboratories have proved the validity and usefulness of both the surgical techniques and the computational set-up for studying cardiovascular functions.

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# The effects of the $\alpha$-adrenoceptor antagonist phentolamine on the responses of the nasal vasculature and nictitating membrane of the cat to cervical sympathetic stimulation

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The nasal vasculature of the dog contains $\alpha$-adrenoceptors (Hall & Jackson, 1968) and receptors of this type have been shown to be present in the nictitating membrane of the cat (Ahlquist, 1948, 1962).

A study was therefore made of the effects of phentolamine on the response of the nasal vasculature and nictitating membrane of 11 cats anaesthetized with pentobarbitone sodium intraperitoneally, to supramaximal preganglionic cervical sympathetic stimulation at frequencies ranging from 0.1 to 25 Hz. A difference in the effect of phentolamine on these tissues was indicated by the unpublished studies of Eccles & Wilson.

Vasoconstriction in the nose was recorded as a reduction of pressure in the sealed nasal cavity (Wilson & Yates, 1975) and contractions of the nictitating membrane were recorded by means of a Statham strain gauge.

Previous experiments had shown that vasoconstriction in the nasal cavity occurred with frequencies as low as 0.1 Hz, the responses increasing with increasing stimulation frequency to reach 80% of the maximum value at approximately 4 Hz and the maximum value at 15 Hz (Eccles & Wilson, 1974). In contrast the contractions of the nictitating membrane increased more slowly. The minimum effective frequency was 0.1 Hz but at 4 Hz the contraction had only attained 45% of the response at 25 Hz, a frequency at which the membrane had still not reached a maximum value. Similar findings were reported by Eccles & Wallis (1976).

Phentolamine was given intravenously in doses of 0.5, 1.0 and 2.0 mg/kg over a period of 1 min to minimize the fall in arterial blood pressure. The responses of both preparations were recorded at 0.2, 1.0, 4.0, 10.0 and 15.0 Hz, frequencies selected to give a representation of the full frequency/response curves. The responses of both preparations were recorded using these frequencies on three occasions before and after phentolamine. Each of the doses of phentolamine reduced the vasoconstriction produced at 15 Hz by approximately 7, 15 and 35% respectively and the contractions of the nictitating membrane by 25, 40 and 70%. Similar reductions in response were also recorded at other frequencies.

Some evidence was obtained to show that the nasal vascular responses recovered from the effects of phentolamine 75 min after the injection of the drug, compared with the nictitating membrane, the responses of which were unchanged at this time.

The smaller reductions in the vasoconstrictor responses compared with the reductions of the nictitating membrane contractions produced by phentolamine may be due to differences in the type of smooth muscle or in the population of $\alpha$ receptors. They may also be due to the sympathomimetic activity of phentolamine (Goodman & Gilman, 1970) which may be more effective on the nasal blood vessels particularly since they are believed to be the most sensitive tissues in the body to adrenaline (Malcomson, 1959).

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# Prostaglandins D and F with highly selective agonist actions

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Prostaglandins (PGs) E$_2$, D$_2$ and F$_{2\alpha}$, although highly potent agonists in many biological systems, exhibit a low selectivity of action. For example, it can be demonstrated that PGD$_2$ has considerable PGE-like activity, causing relaxation of circular smooth muscle in the cardiovascular, respiratory and reproductive systems. Our recent studies have been concerned with detecting prostaglandin analogues which show a higher selectivity than the naturally occurring compounds.

The typical actions of PGF$_{2\alpha}$, including induction of luteolysis, are mimicked by a number of prostaglandins having an aryloxy substituent in the $\omega$-chain (Dukes, Russell & Walpole, 1974; Binder, Bowler, Brown, Crossley, Hutton, Senior, Slater, Wilkinson & Wright, 1974). Of particular interest is ICI 81008 (17,18,19,20-tetranor-16-*m*-trifluoromethylphenoxy PGF$_{2\alpha}$) which we have shown to be of the same order of potency as PGF$_{2\alpha}$ in stimulating both the rabbit oviduct *in vivo* and the rabbit jejunum *in vitro*, and in producing a delayed, bretylium-sensitive, pressor effect in the sheep. However, in the sheep and rabbit ICI 81008 is at least 2000 times less active than PGD$_2$ and at least 30 times less active than PGF$_{2\alpha}$ in producing an immediate, bretylium-insensitive, pressor effect. In contrast, the 13,14-dihydro-15-oxo analogue of PGD$_2$ has one-fifth of the immediate pressor activity of PGD$_2$ in the sheep and rabbit, but is devoid of activity on the previously mentioned PGF-sensitive systems.

By comparing activities on rabbit and sheep blood pressures with those obtained on rabbit oviduct and rabbit jejunum it has been possible to place a number of compounds in order of selectivity as follows: 13,14-dihydro-15-oxo PGD$_2$; PGD$_2$ 15-methyl ether and 15-oxo PGF$_{2\alpha}$; PGD$_2$; PGF$_{2\alpha}$; 13,14-didehydro PGF$_{2\alpha}$; ICI 81008.

It should be noted that 13,14-dihydro-15-oxo PGD$_2$, PGD$_2$ 15-methyl ether and ICI 81008 show very low activity on PGE-sensitive systems such as the guinea-pig ileum *in vitro*. Recently prostacyclin (PGX) has been shown to be an extremely potent inhibitor of human platelet aggregation *in vitro* and to be some 10 times more active than either PGD$_2$ or PGE$_1$ (Bunting, Gryglewski, Moncada & Vane, 1976; Johnson, Morton, Kinner, Gorman, McGuire, Sun, Whittaker, Bunting, Salmon, Moncada & Vane, 1976). In our studies PGD$_2$ 15-methyl ether and 13,14-dihydro-15-oxo PGD$_2$ had very low platelet inhibitory activity being 3000 times and more than 5000 times less active than PGD$_2$ respectively.

These observations will be discussed in relation to the existence of separate prostaglandin receptors.

We wish to thank ICI Pharmaceuticals, U.K., the Upjohn Co., U.S.A., and the Carlo Erba Research Institute, Italy, for generous supplies of prostaglandins.

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# Inhibition of $Na^+ + K^+$-ATPase by quaternary ammonium compounds and stimulation of acetylcholine release

Z. ABDUL-CADER, J. CHARNOCK, JOANNA L. FIELD, J. PARRY & P.T. NOWELL

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It was previously reported that tetraethylammonium (TEA) and edrophonium competitively inhibited the stimulation of brain $Na^+ + K^+$-ATPase produced by $K^+$ (Abdul-Cader, Eaves, Wood & Nowell, 1973). Further work suggested that bretylium, tetrabutylammonium (TBA), hemicholinium ($HC_3$), dimethylquaternary propranolol (DMP) and xylocholine (TM10) were also competitive against $K^+$ since they inhibited the enzyme system more effectively at half-maximal $K^+$ concentration like TEA and edrophonium (Table 1).

**Table 1** Comparison of the inhibitory effects of quaternary ammonium impounds on $Na^+ + K^+$-ATPase activity of Lubrol-extracted enzyme from guinea-pig brain

| | | *% Inhibition of $Na^+ + K^+$-ATPase activity* | | |
|---|---|---|---|---|
| *Drug* | *Concentration (mole/litre)* | *Maximally stimulated (110 mM NaCl/ 10 mM KCl)* | *Half-maximally stimulated by $K^+$ (110 mM NaCl/ 1 mM KCl)* | *Half-maximally stimulated by $Na^+$ (20 mM NaCl/ 10 mM KCl)* |
| TEA Cl | $10^{-1}$ | 44.1 | 85.0 | 26.0 |
| | $10^{-2}$ | 7.5 | 24.0 | NSE |
| Edrophonium Cl | $10^{-2}$ | 33.9 | 85.0 | 68.0 |
| | $10^{-3}$ | 4.1 | 32.0 | 11.0 |
| Bretylium tosylate | $10^{-3}$ | 6.5 | 46.3 | 5.6 |
| | $10^{-4}$ | 7.0 | 13.4 | NSE |
| TBA Cl | $10^{-3}$ | 22.7 | 51.0 | 7.0 |
| | $10^{-4}$ | NSE | 17.2 | NSE |
| $HC_3Br$ | $10^{-4*}$ | NSE | 26.9 | NSE |
| DMP Cl | $2.5 \times 10^{-4*}$ | 30.1 | 42.0 | 14.2 |
| | $10^{-4}$ | 13.2 | 20.0 | NSE |
| TM10 Br | $10^{-3}$ | 14.9 | 66.0 | 29.2 |
| | $10^{-4}$ | NSE | 29.8 | 9.4 |

Experimental details as described by Shirachi, Allard & Trevor (1970) and Abdul-Cader, Eaves, Wood & Nowell (1973). Results expressed as means of triplicate determinations NSE = no significant effect.

* Higher concentrations could not be used due to interference with the colorimetic method.

In view of reports that conditions leading to inhibition of $Na^+ + K^+$-ATPase enhance acetylcholine release (Paton, Vizi & Zar, 1971; Vizi, 1972), the above drugs were tested for effects on acetylcholine output from rat brain cortical slices using the method of Vizi (1972) with minor modifications. Acetylcholine output during exposure to the drug for 10 min was compared with the mean control output for the same slices during the previous 20 minutes. TEA ($10^{-4}$ M), edrophonium ($10^{-4}$ M) and DMP ($10^{-5}$ M) significantly increased acetylcholine output from $5.3 \pm 0.5$–$7.9 \pm 0.5$ ng $g^{-1}$ $min^{-1}$ ($P < 0.01$), $5.3 \pm 0.4$–$8.3 \pm 0.4$ ng $g^{-1}$ $min^{-1}$ ($P < 0.001$) and $5.9 \pm 0.7$–$8.1 \pm 0.5$ ng $g^{-1}$ $min^{-1}$ ($P < 0.02$) respectively (± s.e. mean; $n = 6$; paired 't' test); $P = 0.03$ by Wilcoxon ranking test. The output returned to the control level when the drugs were washed out. In comparison, ouabain ($10^{-5}$ M) increased the output from $6.2 \pm 0.6$–$18.6 \pm 2.8$ ng $g^{-1}$ $min^{-1}$ (± s.e. mean; $n = 6$) and it remained at approximately this level for 10 min after washing out.

Acetylcholine output generally became irregular with occasional high discharges when the slices were exposed for longer periods to TEA, edrophonium, bretylium, TBA, DMP and TM10 in the $10^{-5}$–$10^{-4}$ M concentration range and $HC_3$ ($10^{-4}$ M), whereas $HC_3$ ($10^{-5}$M) appeared to decrease output. Lowering the $K^+$ concentration to 1.9 mM also produced erratic effects with a high average output of $12.7 \pm 0.8$ ng $g^{-1}$ $min^{-1}$ (± s.e. mean; $n = 9$) over 60 min and in one of these experiments exposure to edrophonium ($10^{-5}$ M) for 30 min resulted in an output of approximately 1 μg $g^{-1}$ $min^{-1}$ pver a 10 min period.

Work is currently in progress to explore the possibility which arises that quaternary ammonium compounds may enhance acetylcholine release by inhibiting the stimulation of $Na^+ + K^+$-ATPase produced by $K^+$.

We should like to thank G.D. Searle & Co., Chicago, for the generous supply of dimethylquaternary propranolol.

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# Detection of small amounts of prostaglandin (PG)-like material and rabbit aorta contracting substance (RCS) released into the blood of the rat

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The cascade superfusion technique (Finkleman, 1930; Vane, 1964) is widely used for the detection and bioassay of biologically active substances. The method has also been employed to detect the release of vasoactive substances into the circulation of anaesthetized animals (Vane, 1964, 1969). However, because of the relatively high flow-rates required, the blood-bathed organ technique is very difficult to use with small animals.

Recently, Ferreira & de Souza Costa (1976) have described a laminar flow superfusion technique which can be used to detect very small amounts of biologically active material with very slow rates of superfusion and this latter apparatus has now been adapted for the blood-bathed organ technique in rats, as suggested by Ferreira & de Souza Costa (1976).

The apparatus is shown in Figure 1. Blood is removed at 0.1 ml/min through a polyethylene cannula in a carotid artery and pumped directly over a rabbit aorta and rat stomach strip, set up in a way to be described elsewhere, Krebs (gassed with 95% $O_2$ + 5% $CO_2$) containing antagonists (Gilmore, Vane & Wyllie, 1968), is simultaneously passed over the tissues. This Krebs flow is initially set at 0.2 ml/min, then reduced to 0.1 ml/min as the blood reaches the tissues, in order to maintain a constant flow over the tissues. Standard solutions are injected directly over the tissues and plasma volume is maintained by i.v. injection of 6% dextran/saline until the animal dies (up to 1 hour).

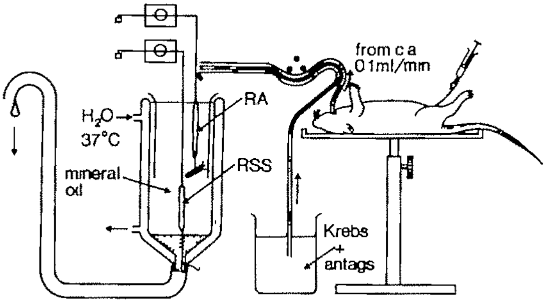

**Figure 1** Blood-bathed organ technique in the rat All tubing is kept as short as possible to reduce temperature changes and the rat is moved as close as possible to the organ bath, using an adjustable operating table RA = rabbit aorta, RSS = rat stomach strip, c.a. = carotid artery.

Using this technique we have detected the release of RCS and PG-like material into the blood, following i.v. injection (into femoral or tail vein) of 0.5 mg/kg of pig pancreatic phospholipase $A_2$. With the use of appropriate tissues, it should also be possible to detect the release of other biologically active substances into the circulation of the rat.

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## A versatile device which resets drifting baselines

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Many biological recordings suffer baseline drift and necessitate regular resetting of the pen recorder. The unit described here will perform such a reset whenever an external positive pulse is applied to the reset input or when the contacts of the manual reset switch are closed (e.g. by an external timer).

The circuit diagram is shown in Figure 1. The differential amplifier has a constant impedance, variable off-set voltage circuit in its inverting input. The potentiometer (VR1) is driven by the servo motor (M) and small gear box (as used in radio controlled models), to produce zero volts at the signal output when the baseline reset is activated. When the auto reset input is positive, transistor Tr 1 switches on, which results in analog switch A conducting and switch B turning off. The servo amplifier is then looking at the signal output. If this is positive the output of the servo amplifier goes positive, transistor Tr 3 switches on, the motor is driven and rotates VR1 such that the signal output approaches zero volts. At this point the servo amplifier output will also be at zero volts, both transistors Tr 2 and Tr 3 will be switched off and the motor will be stationary. If the signal output is negative this results in transistor Tr 2 turning on and the motor rotates in the opposite direction. When not resetting, the servo amplifier input is grounded.

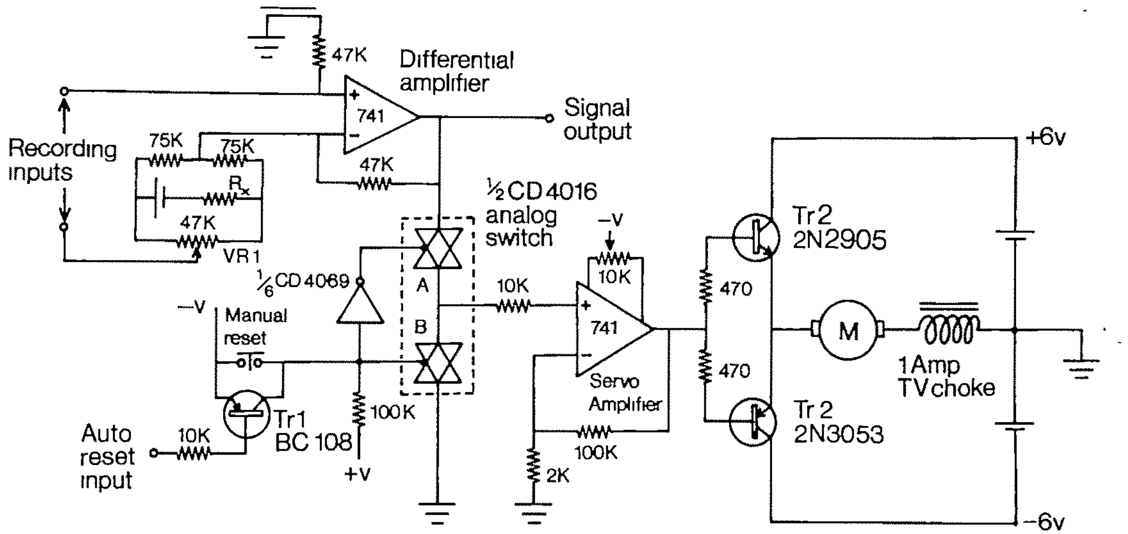

**Figure 1** Circuit diagram of device to automatically reset drifting baselines. Rx is chosen for range of off-set required.

# Irreversible protein binding of [$^{14}$C]-practolol metabolite(s) to hamster liver microsomes

T.C. ORTON & C. LOWERY (introduced by W.E. LINDUP)

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The toxic effects of some chemically stable drugs, e.g. paracetamol, phenacetin and isoniazid, correlate with their metabolism by the microsomal mixed function oxidases via transient reactive intermediates which bind irreversibly to tissue proteins (Gillette, Mitchell & Brodie, 1974).

Practolol is a cardioselective $\beta$-blocking drug which can give rise, in a small proportion of patients, to characteristic skin and eye lesions and sclerosing peritonitis (Waal-Manning, 1975). As some mechanistic hypotheses have involved an immunological reaction (Amos, Brigden & McKerron, 1975; Behan, Behan, Zacharias & Nicholls, 1976), we have investigated the reactivity of practolol and its metabolites with tissue and serum proteins in order to evaluate its potential to form a possible antigenic complex.

[$^{14}$C]-practolol (nuclear labelled) was incubated in air with hamster liver microsomes in the presence of NADPH. The irreversible binding of reactive [$^{14}$C]-intermediates to microsomal proteins was determined by liquid scintillation counting after exhaustive solvent extraction of the microsomes (Potter, Davis, Mitchell, Jollow, Gillette & Brodie, 1973). Terminal metabolites were separated by thin layer chromatography and characterized by co-chromatography with authentic standards.

**Table 1** Inhibition of the irreversible binding of practolol metabolite(s) to hamster liver microsomes *in vitro*

| *Reaction mixture* | *Practolol bound nmol/mg protein/ 30 min* | *% control* |
|---|---|---|
| Complete | 1.49 ± 0.04* | 100 |
| −NADPH | 0.09 ± 0.01 | 6.0 |
| −$O_2$ (100% $N_2$ atmosphere) | 0.46 ± 0.03 | 30.8 |
| +Carbon monoxide (90%, CO, 10% $O_2$) | 0.26 ± 0.02 | 17.4 |
| +Glutathione (10 mM) | 0.32 ± 0.04 | 21.5 |
| +Human Serum Albumin (12.5 mg) | 0.05 ± 0.03 | 3.4 |

* Each value is the mean ± s.d. of 3 or more determinations.

Reaction mixtures consisting of $^{14}$C-practolol (1 mM : 1 μCi), microsomes (5 mg protein), NADPH (0.8 mM) in 0.1 M phosphate buffer pH 7.4 were incubated at 37° for 30 minutes.

The characteristics of the binding reaction of [$^{14}$C]-practolol metabolite(s) to hamster liver microsomes is shown in Table 1. The binding was dependent on oxygen and NADPH and was sensitive to carbon monoxide. The binding was inhibited by glutathione and human serum albumin. [$^{14}$C]-practolol metabolite(s) were bound to the albumin. The rate of binding was linear up to 60 min (5 mg protein per incubation) and 10 mg protein (30 min reaction). The kinetic constants were $V_{max} = 0.08$ nmol bound mg microsomal protein$^{-1}$ min$^{-1}$ and $K_m = 0.06$ mM. The rate of microscomal binding was not altered following pretreatment of hamsters with practolol (50 mg/kg p.o. for 6 days) or phenobarbitone sodium (75 mg/kg i.p. for 3 days). Acetyl labelled [$^{14}$C]-practolol resulted in less binding than nuclear labelled [$^{14}$C]-practolol (0.71 nmol and 1.49 nmol bound mg protein$^{-1}$ 30 min$^{-1}$ respectively).

The major terminal metabolites formed *in vitro* were desacetylpractolol and 3-hydroxypractolol. The formation of 3-hydroxypractolol was NADPH dependent and the maximal rate was 6 nmol mg protein$^{-1}$ 30 min$^{-1}$. [$^{14}$C]-desacetylpractolol *per se* did not combine irreversibly with liver microsomal protein or human serum albumin. Under the same reaction conditions (1 mM substrate, 5 mg protein, 30 min reaction) the rate of the NADPH dependent binding of [$^{14}$C]-desacetylpractolol to hamster liver microsomes was eight times greater than for [$^{14}$C]-practolol.

The possible relevance of the *in vitro* binding of practolol metabolite(s) to mammalian liver microsomes and human serum albumin to the adverse reactions found in man has not yet been established.

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The following demonstration, presented to the British Pharmacological Society Meeting at Mill Hill (5th–7th January, 1977), appeared as a title only in the March 1977 issue of the British Journal of Pharmacology.

## Do 5-HT neurones support self-stimulation?

T.J. CROW & J.F.W. DEAKIN

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With electrodes implanted in certain areas of the brain, rats can be trained to press a lever to deliver electrical stimulation through the implanted electrode (Olds & Milner, 1954). This 'self-stimulation' behaviour presumably indicates that the electrode activates neural systems which have rewarding effects. Previous pharmacological and biochemical evidence suggests that activation of catecholamine-containing systems plays a crucial role in reward mechanisms. In particular it has been found that there are foci of self-stimulation sites at the level of origin of a major dopamine-containing system in the ventral mesencephalon (Crow, 1972) and a noradrenergic system in the nucleus locus coeruleus (Crow, Spear & Arbuthnott, 1972).

More recently it has been found that self-stimulation can also be obtained with electrodes implanted in the region of the median and dorsal raphé nuclei which comprise 5-HT containing cell bodies giving rise to ascending fibres which innervate large areas of the forebrain with tryptaminergic terminals. This finding raises the question of whether the neurochemical mechanisms of reward are more complex than previously suggested. With electrodes on the median raphé nucleus, Miliaressis, Bouchard and Jacobowitz (1975) have claimed that self-stimulation responding is selectively suppressed by administration of the 5-HT synthesis inhibitor *p*-chlorophenylalanine. In these experiments we have attempted to replicate this effect and to study the time course of inhibition following *p*-chlorophenylalanine. We also investigated whether the inhibition is a function of responding by including a rest-pause of 30 min in each test session.

The results (Figure 1) show that there is a modest reduction in responding following *p*-chlorophenylalanine administration but that this effect is maximal at 24 h, when noradrenaline stores are known to be depleted, and that there is considerable recovery by 72 h when 5-HT depletion is greatest. The decline of responding during the test session, and recovery after the rest-pause are greater at 24 and 48 h than at 72 h, findings which also raise doubts as to whether 5-HT depletion is responsible for the inhibition of responding seen in these experiments.

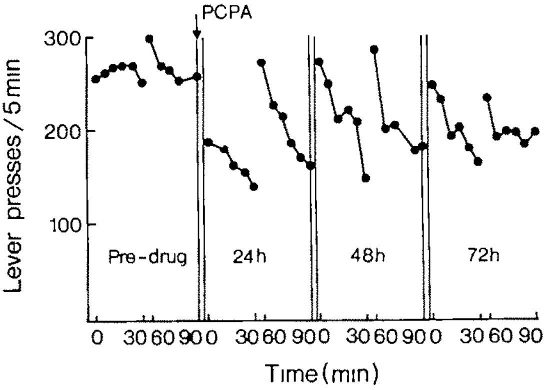

Figure 1

Thus even though electrical self-stimulation can be obtained with electrodes located in the raphé nuclei the role of 5-HT neurones in reward processes appears doubtful.

J.F.W.D. is a Medical Research Council Fellow.

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# ROLE OF 5-HYDROXYTRYPTAMINE IN MORPHINE-, PETHIDINE-, AND METHADONE-INDUCED HYPOTHERMIA IN RATS AT LOW AMBIENT AND ROOM TEMPERATURE

T. OKA

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1 The effect of morphine (10 or 20 mg/kg s.c.), pethidine (25 or 50 mg/kg s.c.) or methadone (4 or 8 mg/kg s.c.) on the body temperature of nontreated and *p*-chlorophenylalanine-pretreated rats was studied at room (21 ± 0.2°C) or low ambient (12 ± 0.2°C) temperature.

2 Neither pethidine nor smaller doses of morphine and methadone altered the mean rectal temperature of rats kept at room temperature but larger doses of morphine and methadone produced significant hypothermia.

3 All narcotic analgesics at doses used in the present investigation produced significant hypothermia in rats maintained in a low ambient temperature. The hypothermia was prevented by naloxone (1 mg/kg s.c.).

4 The administration of *p*-chlorophenylalanine (PCPA, 320 mg/kg i.p.) 48 h before the narcotic injection prevented the fall in body temperature both at room and low ambient temperature.

5 The administration of narcotic analgesics at doses, which when administered by themselves did not alter the body temperature of rats, produced significant hyperthermia in rats pretreated with PCPA.

6 When rats pretreated with PCPA were given 5-hydroxytryptophan (75 mg/kg s.c.) 30 min before narcotic administration, the usual response to narcotics was restored.

7 It appears that pethidine and methadone as well as morphine have both hyperthermic and hypothermic actions in rats and that 5-hydroxytryptamine may be involved in the narcotic-induced hypothermia not only at room temperature but also at low ambient temperature.

## Introduction

The involvement of 5-hydroxytryptamine (5-HT) in the morphine-induced hypothermia of rats at room temperature has already been documented (Oka & Nozaki, 1970; Haubrich & Blake, 1971; Oka, Nozaki & Hosoya, 1972; Samanin, Kon & Garattini, 1972). There have also been several reports which suggest the participation of 5-HT in the decrease in locomotor activity of rats induced not only by morphine (Oka & Nozaki, 1970; Eidelberg & Schwartz, 1970; Buxbaum, Yarbrough & Carter, 1973; Oka & Hosoya, 1976a), but also by pethidine or methadone (Oka & Hosoya, 1976b). Thus, it is of interest to investigate the role of 5-HT in the hypothermia induced by the administration of pethidine or methadone to rats. In initial experiments it was found that neither pethidine nor a small dose of morphine or methadone produced hypothermia in rats at room temperature; however, significant hypothermia was produced at a lower ambient temperature. Therefore, the present experiments were performed both at room temperature and at a low ambient temperature.

## Methods

Male Wistar rats weighing between 170 and 250 g were used in the present study. Body temperature was measured as described previously (Oka *et al.*, 1972), with a thermistor probe inserted through the anus for a distance of 6 cm and taped to the base of the tail. Animals were placed in individual cages and could move relatively freely during the experiment. The interior of the experimental chamber was maintained at either 12 ± 0.2°C or 21 ± 0.2°C with 50 ± 2% relative humidity. Rectal temperatures were continuously recorded on Hokushin E36N1 P313 recorders. All experiments were begun at 10 h 00 min.

Morphine hydrochloride was bought from Sankyo Company (Tokyo, Japan), pethidine hydrochloride from Tanabe Seiyaku Co. (Osaka, Japan), and DL-*p*-chlorophenylalanine (PCPA) and 5-hydroxy-DL-tryptophan (5-HTP) from Nakarai Chemicals (Kyoto, Japan). Naloxone hydrochloride was a gift from Sankyo Co. Methadone hydrochloride was kindly supplied by Nippon Roche Research Center (Kamakura, Japan).

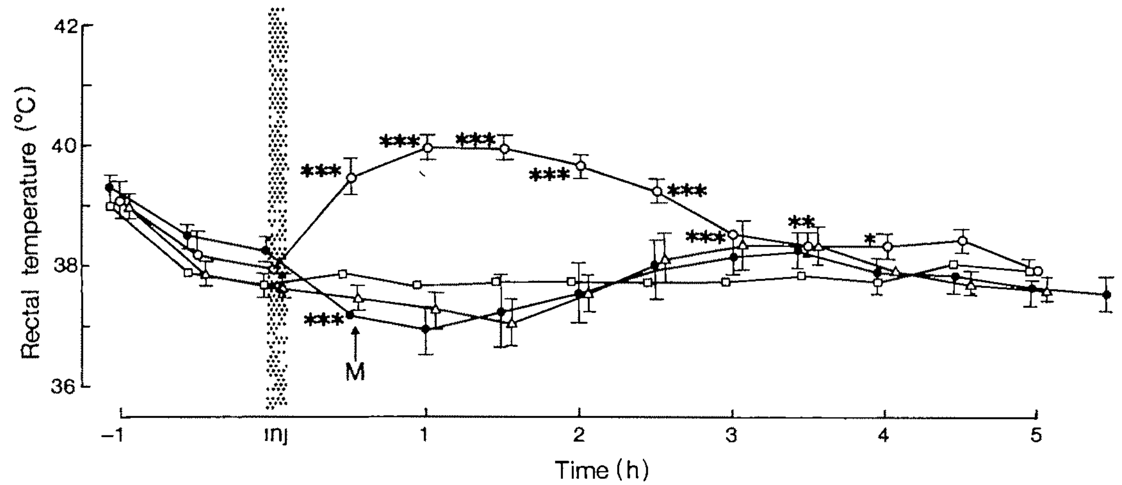

**Figure 1** The effect of morphine on the mean rectal temperature of rats pretreated with *p*-chlorophenylalanine (PCPA); ambient temperature 21 °C Measurements of body temperature were begun immediately after the thermistor probe was inserted into the rectum. Saline (5 ml/kg), morphine (10 mg/kg) or 5-hydroxytryptophan (5-HTP, 75 mg/kg) was administered (at stippled bar) subcutaneously 1 h after inserting the thermistor probe, except for one group in which morphine (M) was given (indicated by arrow) 30 min after 5-HTP administration to PCPA pretreated rats. PCPA (320 mg/kg) was injected 48 h before stippled bar. Symbols and vertical bars represent means with s.e. mean. (□) saline, $n=6$; (△) morphine, $n=6$; (○) PCPA+ morphine, $n=6$; (●) PCPA+5-HTP+morphine, $n=6$.

*, $P<0.05$; **, $P<0.02$; ***, $P<0.01$ when compared with values of a group that had received saline.

All drugs except PCPA were dissolved in sterile 0.9% NaCl solution (saline) and were administered subcutaneously or intraperitoneally in volumes of 5 ml/kg. All drug dosages are expressed in terms of their salts. PCPA was adminsitered as a very fine saline suspension.

All data were statistically analyzed by Student's *t* test.

## Results

After the thermistor probes had been inserted and the rats placed in the experimental chamber, a period of 1 h elapsed before their body temperatures stabilized between 37.5 and 38.5°C. Thereafter, their body temperatures remained constant over the next 5 h both at room and at low ambient temperatures (Figures 1 and 2). Accordingly, test substances were administered 1 h after insertion of the thermistor probe.

### *Effects of narcotic analgesics on the body temperature of rats at room temperature and its modification by pretreatment with* p*-chlorophenylalanine and 5-hydroxytryptophan*

Effects of narcotic analgesics on body temperature of rats placed at a room temperature of 21±0.2°C with 50±2% relative humidity are illustrated in Figure 1 and Tables 1, 2 and 3.

The subcutaneous administration of morphine (10 mg/kg), pethidine (25 or 50 mg/kg) or methadone (4 mg/kg) alone had almost no effect on the mean rectal temperature of rats. In contrast, methadone (8 mg/kg) produced a significant fall in the body temperature (Table 3). Morphine (20 mg/kg), on the other hand, induced a hypothermic response followed by a hyperthermic response in rats (Table 1). Pretreatment of rats with PCPA (320 mg/kg, i.p.) 48 h before narcotic administration not only blocked the hypothermic response to morphine (20 mg/kg) but converted it to a significant hyperthermic response (Table 1). Pretreatment with PCPA did not block the hypothermic response to methadone (8 mg/kg) at 1 h but blocked it at 2 h and the animals exhibited a significant hyperthermic response at 3 h after methadone injection (Table 3). The administration of morphine (10 mg/kg), pethidine (25 or 50 mg/kg) or methadone (4 mg/kg) to PCPA pretreated rats produced prominent hyperthermia (Figure 1 and Tables 2 and 3). When rats pretreated with PCPA were given 5-hydroxytryptophan (5-HTP) (75 mg/kg, s.c.) 30 min before morphine administration, a typical hyperthermic response to morphine in PCPA-pretreated rats was no longer seen (Figure 1). Although 5-HTP alone produced a marked fall in the body temperature of rats pretreated with PCPA

**Table 1** The effect of morphine on the mean rectal temperature of rats and its modification by *p*-chlorophenylalanine (PCPA); ambient temperature 21 °C

| *Treatment* | n | *0 h* | *1 h* | *2 h* | *3 h* | *4 h* |
|---|---|---|---|---|---|---|
| Saline | 6 | 37.7<br>±0.2 | 37.7<br>±0.1 | 37.8<br>±0.1 | 37.8<br>±0.1 | 37.8<br>±0.1 |
| Morphine | 6 | 37.7<br>±0.3 | †<br>36.5<br>±0.5 | †<br>35.3<br>±1.0 | 36.4<br>±0.8 | ††<br>38.7<br>±0.2 |
| PCPA | 6 | 38.0<br>±0.2 | 38.1<br>±0.2 | 38.2<br>±0.3 | 38.3<br>±0.3 | 38.1<br>±0.2 |
| PCPA+<br>morphine | 6 | 38.1<br>±0.2 | †<br>38.9<br>±0.5<br>*** | †††<br>39.6<br>±0.3<br>*** | †††<br>38.8<br>±0.2<br>** | †<br>38.3<br>±0.2 |

Either saline (5 ml/kg) or morphine (20 mg/kg) was administered subcutaneously at 0 h. PCPA (320 mg/kg) was injected intraperitoneally 48 h before 0 h The body temperature measurements were begun one hour before 0 h. Values before 0 h of each treatment are omitted here since they were essentially the same as data depicted in Figure 1. Data of a group which had received saline are those depicted in Figure 1, but are included here for ease of comparison. Values are the mean with s.e. mean. *n* = number of animals tested.

†, $P<0.05$; ††, $P<0.02$; †††, $P<0.01$ when compared with values of a group which had received saline. **, $P<0.02$; ***, $P<0.01$ comparing PCPA+morphine with morphine alone.

**Table 2** The effect of pethidine on the mean rectal temperature of rats and its modification by *p*-chlorophenylalanine (PCPA); ambient temperature 21 °C

| *Treatment* | n | *0 h* | *1 h* | *2 h* | *3 h* | *4 h* |
|---|---|---|---|---|---|---|
| Saline | 6 | 37.7<br>±0.2 | 37.7<br>±0.1 | 37.8<br>±0.1 | 37.8<br>±0.1 | 37.8<br>±0.1 |
| Pethidine (25 mg/kg) | 6 | 37.7<br>±0.2 | 37.4<br>±0.3 | 37.7<br>±0.2 | 37.7<br>±0.2 | 37.5<br>±0.2 |
| Pethidine (50 mg/kg) | 6 | 37.7<br>±0.3 | 37.6<br>±0.4 | 37.2<br>±0.3 | 37.3<br>±0.3 | 37.7<br>±0.2 |
| PCPA+ pethidine (25 mg/kg) | 6 | 38.2<br>±0.4 | ††<br>39.0<br>±0.5<br>** | †<br>38.8<br>±0.3<br>** | 38.4<br>±0.2<br>* | 38.2<br>±0.3 |
| PCPA+ pethidine (50 mg/kg) | 6 | 38.0<br>±0.2 | †††<br>39.0<br>±0.3<br>*** | 38.1<br>±0.3<br>* | 37.7<br>±0.2 | 37.8<br>±0.2 |
| PCPA+ 5-HTP | 6 | 38.3<br>±0.2 | †††<br>36.6<br>±0.3 | ††<br>37.1<br>±0.2 | 37.4<br>±0.3 | 37.5<br>±0.2 |
| PCPA+ 5-HTP+ pethidine (25 mg/kg) | 6 | 38.2<br>±0.2 | †††<br>35.8<br>±0.3 | †††<br>35.8<br>±0.2<br>° ° ° | †††<br>36.2<br>±0.4<br>° | ††<br>36.8<br>±0.4 |

Either pethidine or 5-hydroxytryptophan (5-HTP, 75 mg/kg) was administered subcutaneously at 0 h except in the experiment, listed at the foot of the table, in which pethidine was injected at 0.5 h (i.e., 30 min after 5-HTP). PCPA (320 mg/kg) was injected intraperitoneally 48 h before 0 h. Values of a group that had received saline are those depicted in Figure 1. Values are the mean with s.e. mean. *n* = number of animals tested.

†, $P<0.05$; ††, $P<0.02$; †††, $P<0.01$ when compared with values of a group that had received saline. *, $P<0.05$; **, $P<0.02$; ***, $P<0.01$ comparing PCPA+narcotic with corresponding narcotic alone. °, $P<0.05$; ° ° °, $P<0.01$ comparing PCPA+5-HTP+pethidine with PCPA+5-HTP.

**Table 3** The effect of methadone on the mean rectal temperature of rats and its modification by *p*-chlorophenylalanine (PCPA); ambient temperature 21 °C

| *Treatment* | n | *0 h* | *1 h* | *2 h* | *3 h* |
|---|---|---|---|---|---|
| Saline | 6 | 37.7<br>±0.2 | 37.7<br>±0.1 | 37.8<br>±0.1 | 37.8<br>±0.1 |
| Methadone (4 mg/kg) | 6 | 37.8<br>±0.2 | 36.7<br>±0.8 | 38.0<br>±0.3 | 38.1<br>±0.2 |
| Methadone (8 mg/kg) | 6 | 37.8<br>±0.1 | †††<br>35.1<br>±0.2 | †††<br>34.4<br>±0.8 | 37.0<br>±0.5 |
| PCPA+ methadone (4 mg/kg) | 6 | 38.1<br>±0.3 | †††<br>39.2<br>±0.2<br>** | ††<br>38.8<br>±0.3 | 38.1<br>±0.3 |
| PCPA+ methadone (8 mg/kg) | 7 | 38.1<br>±0.1 | †††<br>35.8<br>±0.4 | 37.8<br>±0.5<br>*** | †<br>38.3<br>±0.1<br>* |
| PCPA+ 5-HTP+ methadone (4 mg/kg) | 6 | 38.2<br>±0.3 | †††<br>35.9<br>±0.3 | †††<br>35.8<br>±0.4<br>°° | 37.1<br>±0.4 |

Either methadone or 5-hydroxytryptophan (5-HTP, 75 mg/kg) was administered subcutaneously at 0 h except in the experiment, listed at the foot of the table, in which methadone was injected at 0.5 h (i.e., 30 min after 5-HTP). PCPA (320 mg/kg) was injected intraperitoneally 48 h before 0 h. Values of a group that received saline are those depicted in Figure 1. Values are the mean with s.e. mean. $n$ = number of animals tested.
†, $P<0.05$; ††, $P<0.02$; †††, $P<0.01$ when compared with values of a group that had received saline. *, $P<0.05$; **, $P<0.02$; ***, $P<0.01$ comparing PCPA+narcotic with corresponding narcotic alone. °°, $P<0.02$ comparing PCPA+5-HTP+methadone with PCPA+5-HTP.

**Table 4** The effect of morphine on the mean rectal temperature of rats and its modification by *p*-chlorophenylalanine (PCPA); ambient temperature 12°C

| *Treatment* | n | *0 h* | *0.5 h* | *1 h* | *2 h* | *3 h* |
|---|---|---|---|---|---|---|
| Saline | 6 | 38.0<br>±0.1 | *38.2*<br>±0.1 | 38.0<br>±0.2 | 37.9<br>±0.3 | 38.1<br>±0.2 |
| Morphine | 6 | 37.9<br>±0.2 | †††<br>37.2<br>±0.3 | 36.8<br>±0.5 | 36.5<br>±0.8 | 37.9<br>±0.4 |
| PCPA | 6 | 38.2<br>±0.2 | 38.0<br>±0.2 | 38.1<br>±0.2 | 38.3<br>±0.1 | 38.1<br>±0.1 |
| PCPA+ morphine | 7 | 38.1<br>±0.2 | 38.0<br>±0.2 | 38.4<br>±0.5<br>* | †<br>38.7<br>±0.2<br>** | 38.5<br>±0.2 |

Morphine (10 mg/kg) was administered subcutaneously at 0 h. PCPA (320 mg/kg) was injected intraperitoneally 48 h before 0 h. Values of a group that had received saline are those depicted in Figure 2. Values are the mean with s.e. mean. $h$ = number of animals tested.
†, $P<0.05$; †††, $P<0.01$ when compared with values of a group received saline. *, $P<0.05$; **, $P<0.02$ comparing PCPA+morphine with morphine alone.

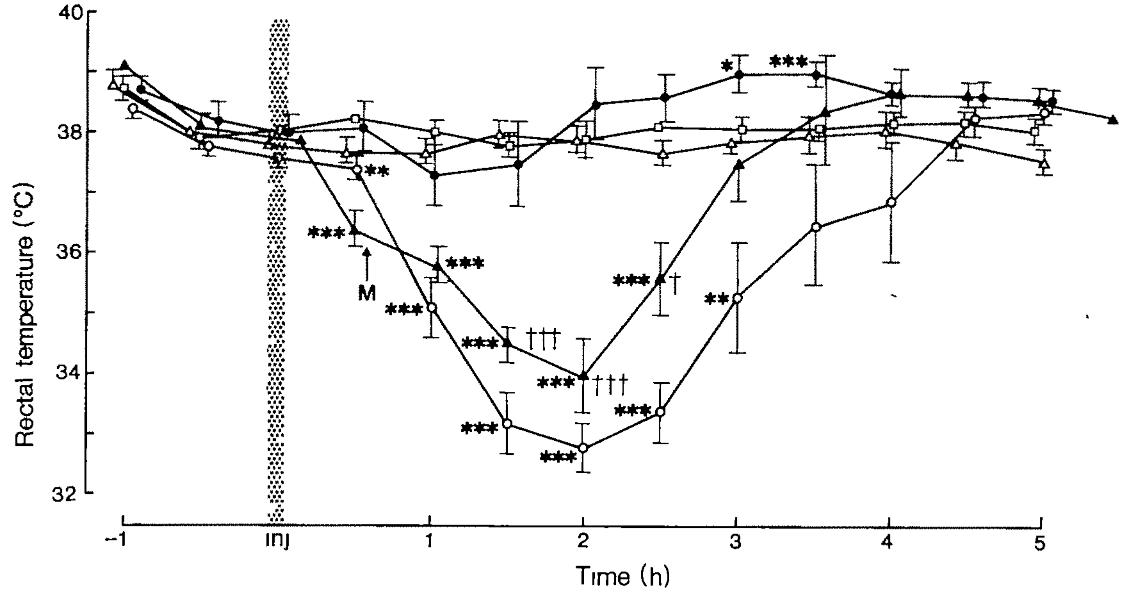

**Figure 2** The effect of morphine on the mean rectal temperature of rats and its modification by *p*-chlorophenylalanine (PCPA), 5-hydroxytryptophan (5-HTP) and naloxone at 12°C. Acute administration of drugs or saline at stippled bar. One group of rats ($n=6$) received saline (5 ml/kg, s.c., □). Another group ($n=6$) received morphine (20 mg/kg s.c., ○). A third group ($n=8$) was pretreated with PCPA (320 mg/kg i.p.) 48 h before morphine administration (●). A fourth group ($n=6$) was pretreated with PCPA 48 h before 5-HTP (75 mg/kg, s.c.) administration (at stippled bar) and then received morphine (M, at arrow) 30 min after 5-HTP administration (▲). A fifth group ($n=6$) was pretreated with naloxone (1 mg/kg s.c.) immediately before morphine administration (△). Symbols and vertical bars represent means with s.e. mean.

*, $P<0.05$; **, $P<0.02$; ***, $P<0.01$ when compared with values of a group received saline. †, $P<0.05$; †††, $P<0.01$ comparing PCPA+5-HTP+morphine with PCPA+5-HTP.

(Table 2), body temperatures of a group that had received PCPA, 5-HTP and morphine were not significantly different from animals that had received PCPA and 5-HTP. On the other hand, the body temperatures of a group given PCPA, 5-HTP and pethidine or methadone were significantly lower than those injected with PCPA and 5-HTP (Tables 2 and 3).

### *Effects of narcotic analgesics on the body temperature of rats at low ambient temperature and its modification by pretreatment with* p*-chlorophenylalanine and 5-hydroxytryptophan*

Values of the mean rectal temperature of control (saline-treated) rats kept at a low ambient temperature (12°C) were essentially the same as those kept at room temperature throughout the experimental period (Figures 1 and 2). Data of PCPA pretreated rats exposed to a low environmental temperature were also not significantly different from those kept at room temperature (Tables 1 and 4). Effects of narcotic analgesics on body temperature of rats maintained at a low ambient temperature, however, were clearly different from those kept at room temperature. All narcotics produced a significant fall in body temperature of rats maintained in an ambient temperature of 12±0.2°C with 50±2% relative humidity (Figure 2 and Tables 4, 5 and 6). Pretreatment of rats with naloxone (1 mg/kg, s.c.), a specific narcotic antagonist, immediately before narcotic administration prevented the morphine-induced hypothermia (Figure 2) and greatly reduced the hypothermic effects of pethidine and methadone (Tables 5 and 6). The intraperitoneal administration of 320 mg/kg of PCPA 48 h before the narcotic injection not only reduced the hypothermic response to narcotics (Figure 2 and Tables 4, 5 and 6), but the animals exhibited significant hyperthermic response to morphine or to a lower dose of methadone (Figure 2 and Table 6). When rats pretreated with PCPA were given 5-HTP 30 min before administration of morphine, a typical hypothermic response to morphine was restored (Figure 2). The administration of 5-HTP alone to PCPA pretreated rats produced a fall in body temperature (Table 5). However, the body temperatures of a group which had received PCPA, 5-HTP and either morphine, pethidine or methadone were significantly lower than those which received PCPA and 5-HTP (Figure 2 and Tables 5 and 6).

## Discussion

The results of the present investigation suggest that narcotic analgesics such as morphine, pethidine and methadone are likely to have both stimulant and depressant actions on the body temperature of rats and that 5-HT may be involved in the narcotic-induced hypothermia not only at room temperature but also at low ambient temperature.

The involvement of 5-HT in narcotic-induced hypothermia is suggested by the fact that the usual marked hypothermic response of rats to narcotic analgesics is inhibited by the pretreatment of the animals with PCPA, which depletes 5-HT stores (Koe & Weissman, 1966). Additionally, the administration of 5-HTP, a 5-HT precursor, to the animals pretreated with PCPA restored the typical hypothermic response to narcotic analgesics. Moreover, hypothermia can be

**Table 5** The effect of pethidine on the mean rectal temperature of rats and its modification by naloxone or *p*-chlorophenylalanine (PCPA); ambient temperature 12°C

| *Treatment* | n | *0 h* | *1 h* | *2 h* | *3 h* | *4 h* | *5 h* |
|---|---|---|---|---|---|---|---|
| Saline | 6 | 38.0<br>±0.1 | 38.0<br>±0.2 | 37.9<br>±0.3 | 38.1<br>±0.2 | 38.2<br>±0.2 | 38.1<br>±0.2 |
| Pethidine (25 mg/kg) | 5 | 38.0<br>±0.2 | †††<br>35.7<br>±0.3 | †<br>36.7<br>±0.5 | 37.4<br>±0.4 | 37.7<br>±0.1 | 37.9<br>±0.3 |
| Pethidine (50 mg/kg) | 6 | 37.5<br>±0.3 | †††<br>34.4<br>±0.7 | †††<br>33.6<br>±0.9 | †††<br>34.8<br>±0.8 | ††<br>36.0<br>±0.7 | 36.9<br>±0.6 |
| Naloxone | 6 | 38.4<br>±0.1 | 38.1<br>±0.1 | 38.2<br>±0.1 | 38.1<br>±0.2 | 38.3<br>±0.2 | 38.3<br>±0.1 |
| Naloxone+ pethidine (50 mg/kg) | 6 | 37.9<br>±0.2 | †††<br>36.8<br>±0.3<br>XX | †††<br>36.3<br>±0.3<br>X | †††<br>36.1<br>±0.2 | ††<br>37.2<br>±0.3 | ††<br>37.3<br>±0.2 |
| PCPA+ pethidine (25 mg/kg) | 6 | 38.1<br>±0.1 | 37.8<br>±0.3<br>*** | 37.7<br>±0.3 | 37.8<br>±0.2 | 37.9<br>±0.2 | 38.0<br>±0.1 |
| PCPA+ pethidine (50 mg/kg) | 5 | 38.4<br>±0.2 | †††<br>36.1<br>±0.4<br>* | †††<br>35.9<br>±0.3 | †††<br>36.3<br>±0.3 | 37.7<br>±0.3 | 38.4<br>±0.2 |
| PCPA+ 5-HTP | 5 | 37.9<br>±0.2 | †††<br>36.2<br>±0.4 | †<br>36.8<br>±0.5 | 37.6<br>±0.3 | 38.1<br>±0.1 | 37.9<br>±0.1 |
| PCPA+ 5-HTP+ pethidine (25 mg/kg) | 5 | 37.9<br>±0.1 | †††<br>35.9<br>±0.3 | †††<br>35.5<br>±0.4<br>° | †††<br>36.2<br>±0.3<br>°° | †††<br>37.2<br>±0.2<br>°°° | 37.5<br>±0.4 |

Pethidine, naloxone (1 mg/kg) or 5-hydroxytryptophan (5-HTP, 75 mg/kg) was injected subcutaneously at 0 h except in the experiment, listed at the foot of the table, in which pethidine was injected at 0.5 h (i.e., 30 min after 5-HTP). PCPA (320 mg/kg) was administered intraperitoneally 48 h before 0 h. Values of a group that had received saline are those depicted in Figure 1. Values are the mean with s.e. mean. *n*=number of animals tested.

†, $P<0.05$; ††, $P<0.02$; †††, $P<0.01$ when compared with values of a group that received saline. X, $P<0.05$; XX, $P<0.02$ comparing naloxone+pethidine with pethidine. *, $P<0.05$; ***, $P<0.01$ comparing PCPA+narcotic with corresponding narcotic alone. °, $P<0.05$; °°, $P<0.02$; °°°, $P<0.01$ comparing PCPA+5-HTP+pethidine with PCPA+5-HTP.

shown in rats after intraventricular (Feldberg & Lotti, 1967) or intracisternal (Bruinvels, 1970) administration of 5-HT. Finally, it has been reported that morphine accelerates the turnover rate of 5-HT in rat brain (Yarbrough, Buxbaum & Sanders-Bush, 1973; Goodlet & Sugrue, 1974; Sawa & Oka, 1976), pethidine inhibits the uptake mechanism of 5-HT-containing neurones (Carlsson & Lindqvist, 1969), and methadone inhibits the uptake of [$^3$H]-5-HT by synaptosomes (Ciofalo, 1974).

On the other hand, 5-hydroxytryptamine does not appear to play an important role in the maintenance of body temperature in rats kept both at room and at a low ambient temperature since the administration of PCPA alone had no effect on the body temperature at these environmental temperatures.

A small dose of morphine has been reported to produce hyperthermia in the rat (Hermann, 1942; Cox, Ary, Chesarek & Lomax, 1976), and, even with a large dose of morphine, a prominent hyperthermia can be produced in morphine-tolerant rats (Gunne, 1960; Oka *et al.*, 1972). Thus, morphine may have both hyperthermic and hypothermic effects in rats. Moreover, small, moderate or large doses of morphine produce, respectively, an elevation of, no significant change in or a fall in body temperature of rats. Thus, the apparent lack of effect of moderately large doses of morphine may be due to the simultaneous activation of hyperthermic and hypothermic responses of equal magnitude. If this hypothesis were correct, the hyperthermia induced by the administration of morphine to PCPA pretreated rats may result from the suppresssion of the hypothermic response to morphine. Although a hyperthermic action of

**Table 6** The effect of methadone on the mean rectal temperature of rats and its modification by naloxone or *p*-chlorophenylalanine (PCPA); ambient temperature 12°C

| *Treatment* | n | *0 h* | *0.5 h* | *1 h* | *1.5 h* | *2.5 h* | *5 h* |
|---|---|---|---|---|---|---|---|
| Saline | 6 | 38.0<br>±0.1 | 38.2<br>±0.1 | 38.0<br>±0.2 | 37.8<br>±0.2 | 38.1<br>±0.1 | 38.1<br>±0.2 |
| Methadone<br>(4 mg/kg) | 5 | 37.9<br>±0.2 | †††<br>35.8<br>±0.2 | †††<br>34.7<br>±0.7 | 35.6<br>±1.2 | 37.5<br>±0.3 | 38.3<br>±0.1 |
| Methadone<br>(8 mg/kg) | 7 | 37.7<br>±0.1 | †††<br>35.1<br>±0.2 | †††<br>32.5<br>±0.3 | †††<br>31.6<br>±0.6 | †††<br>34.8<br>±0.7 | 37.7<br>±0.2 |
| Naloxone+ methadone (8 mg/kg) | 6 | 37.9<br>±0.2 | †††<br>37.3<br>±0.1<br>XXX | †††<br>37.1<br>±0.2<br>XXX | 37.3<br>±0.2<br>XXX | 37.9<br>±0.2<br>XXX | 37.8<br>±0.2 |
| PCPA+ metahdone (4 mg/kg) | 6 | 38.1<br>±0.3 | z<br>38.1<br>±0.3<br>*** | ††<br>38.6<br>±0.5<br>*** | 39.0<br>±0.3<br>** | 38.6<br>±0.3<br>* | 38.5<br>±0.1 |
| PCPA+ methadone (8 mg/kg) | 6 | 38.2<br>±0.2 | †††<br>36.2<br>±0.1<br>*** | †††<br>33.5<br>±0.5 | †††<br>32.3<br>±1.5 | 36.3<br>±1.1 | 38.5<br>±0.3<br>* |
| PCPA+ 5-HTP+ methadone (4 mg/kg) | 6 | 38.0<br>±0.3 | †††<br>36.9<br>±0.1 | †††<br>34.3<br>±0.5<br>° ° | †††<br>33.0<br>±0.6<br>° ° ° | †††<br>32.9<br>±0.5<br>° ° ° | 37.4<br>±0.4 |

Methadone, naloxone (1 mg/kg) or 5-hydroxytryptophan (5-HTP, 75 mg/kg) was injected subcutaneously at 0 h except in the experiment, listed at the foot of the table, in which methadone was injected at 0.5 h (i.e., 30 min after 5-HTP). PCPA (320 mg/kg) was administered intraperitoneally 48 h before 0 h. Values of a group that had received saline are those depicted in Figure 1. Values are the mean with s.e. mean. *n* = number of animals tested.

††, $P<0.02$; †††, $P<0.01$ when compared with values of a group that had received saline. XXX, $P<0.01$ comparing naloxone+methadone with methadone alone. *, $P<0.05$; **, $P<0.02$; ***, $P<0.01$ comparing PCPA+ narcotic with corresponding narcotic alone. °°, $P<0.02$; °°°, $P<0.01$ comparing PCPA+5-HTP+methadone with PCPA+5-HTP.

pethidine or methadone on the body temperature of rats has not been reported previously, it is concluded from the results of the present investigation that both pethidine and methadone, as well as morphine, may have biphasic effects on the body temperature of rats since both of these narcotic analgesics also produce hyperthermia in PCPA pretreated rats.

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# EFFECTS OF DIBUTYRYL CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE AND THEOPHYLLINE ON THE BULLFROG SYMPATHETIC GANGLION CELLS

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1 Effects of dibutyryl cyclic adenosine 3′,5′-monophosphate (dibutyryl cyclic AMP) and theophylline on bullfrog sympathetic ganglion cells were examined in order to test the hypothesis that cyclic AMP is essential for the generation of slow inhibitory postsynaptic potentials (i.p.s.ps) in these cells.

2 In the absence or presence of theophylline, dibutyryl cyclic AMP did not hyperpolarize but rather tended to depolarize ganglia that were hyperpolarized by adrenaline.

3 Theophylline augmented neither the P-potential (slow i.p.s.p.) nor adrenaline-induced hyperpolarization.

4 Thus, cyclic AMP does not seem to be essential for the generation of the slow i.p.s.p., at least in amphibian sympathetic ganglion cells.

## Introduction

Slow potential changes associated with synaptic activation of sympathetic ganglia have been reported by many authors and attempts have been made to confirm the suggestion of Eccles & Libet (1961) that a catecholamine may be the mediator of the hyperpolarizing potential. The latter potential is recorded extracellularly as the P-potential and intracellularly as the slow inhibitory postsynaptic potential or slow i.p.s.p. Membrane hyperpolarizations induced by adrenaline and dopamine have been observed in mammalian (Libet & Kobayashi, 1969; Kobayashi & Libet, 1970; Libet & Tosaka, 1970; 1971; Christ & Nishi, 1971; Dun & Nishi, 1974) and amphibian (Nakamura & Koketsu, 1972; Weight, 1973; Libet & Kobayashi, 1974; Koketsu & Nakamura, 1976) sympathetic ganglia. However, the mechanisms involved in the production of the slow i.p.s.p. or these catecholamine-induced hyperpolarizations are not entirely clear.

The main aims of this investigation were to test the hypothesis of McAfee & Greengard (1972) that adenosine 3′,5′-monophosphate (cyclic AMP) plays an essential role in the generation of the slow i.p.s.p. and catecholamine-induced hyperpolarizations of sympathetic ganglia and to compare the ionic mechanisms underlying cyclic AMP-induced changes in membrane potential of ganglion cells with those responsible for the catecholamine-induced responses. On the basis of the work of McAfee & Greengard (1972) dibutyryl cyclic AMP would be expected to induce a hyperpolarization. Kuba & Nishi (1976), on the other hand, reported that dibutyryl cyclic AMP did not hyperpolarize bullfrog sympathetic ganglion cells. Since the latter authors had observed hyperpolarizing responses to caffeine, it was of interest to examine the effect of another methylxanthine, theophylline, which is known to inhibit phosphodiesterase activity and which, therefore, might be expected to enhance the effects of endogenously produced cyclic AMP and its administered dibutyryl derivative.

The present experiments were therefore carried out in order to examine the effect of dibutyryl cyclic AMP and theophylline on bullfrog sympathetic ganglion cells, for the purpose of clarifying whether cyclic AMP is essential for the generation of the slow i.p.s.p. or adrenaline-induced hyperpolarization.

## Method

Paravertebral sympathetic ganglion chains isolated from bullfrogs (*Rana catesbeiana*) were used in the present experiments. The membrane potential changes of sympathetic ganglion cells were recorded from ganglion-postganglionic nerve preparations by use of sucrose-gap methods (Koketsu & Nishi, 1968; Nishi & Koketsu, 1968b; see also Kosterlitz, Lees & Wallis, 1968). The membrane potential changes of ganglion cells were also recorded by intracellular microelectrodes inserted into the ganglion cell-bodies (Nishi & Koketsu, 1960). Microelectrodes were filled with

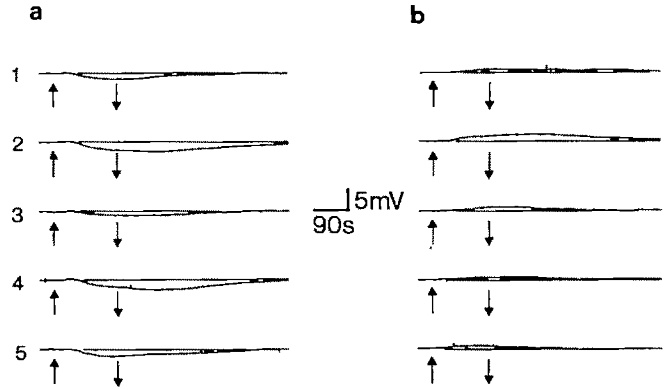

**Figure 1** Effects of (a) adrenaline (adrenaline-induced hyperpolarizations) and (b) dibutyryl cyclic AMP on sympathetic ganglia; all records were obtained by the sucrose-gap method, and moments of application and withdrawal of these drugs are indicated by upward and downward arrows, respectively. (a and b) Records 1, 2 and 3 were obtained from the same preparation, and records 4 and 5 were from nicotine-treated and curarized ganglia, respectively. Records 3 were obtained in the presence of theophylline (2 mM). (a) Adrenaline, 1 mM for record 2, 0.3 mM for other records; (b) dibutyryl cyclic AMP, 3 mM for record 2, 1 mM for other records.

3 M KCl and those with resistances in the range 20–30 megaohms were selected for the present experiment.

Ionic compositions of solutions used in the present experiment were as follows: the Ringer solution (mM): NaCl 112, KCl 2, $CaCl_2$ 1.8 and $NaHCO_3$ 2; the $K^+$-free solution (mM): NaCl 112, $CaCl_2$ 1.8 and $NaHCO_3$ 2. Preparations were continuously superfused with one of the solutions flowing through a preparation-trough (50 x 5 x 4 mm) at the rate of 0.2 ml/second.

Drugs used were as follows: dibutyryl cyclic AMP, sodium (P-L Biochemicals, Inc.), theophylline (Wako Pure Chemical Ind., Japan), (—)-adrenaline bitartrate (SIGMA), nicotine sulphate (Katayama Chemical Ind., Japan), and (+)-tubocurarine chloride ((+)-Tc) (Wako Pure Chemical Ind. Japan).

All experiments were carried out at room temperature (20–22°C).

## Results

### *Effects of dibutyryl cyclic AMP on the membrane potential*

The effect of dibutyryl cyclic AMP on the membrane potential of ganglion cells was tested by use of the sucrose-gap method, in those ganglia in which it was confirmed that adrenaline (0.3 mM) induced a hyperpolarization (cf. Nakamura & Koketsu, 1972; Koketsu & Nakamura, 1976). No hyperpolarization was observed when dibutyryl cyclic AMP (1 mM) was applied to these ganglia (10 preparations); rather, the ganglia tended to depolarize (less than 1 mV) and notable depolarizations (about 2 mV) occurred when bibutyryl cyclic AMP was used in a concentration of 3 mM, which was the highest concentration used in these experiments. Effects of dibutyryl cyclic AMP on the membrane potential were also examined in the presence of nicotine (0.24 mM) or (+)-Tc (0.14 mM) because the slow i.p.s.p. can be recorded in the presence of nicotine or (+)-Tc. Dibutyryl cyclic AMP (1 mM) never hyperpolarized the ganglion cells in these nicotinized or curarized ganglia. Examples of these experiments are shown in Figure 1.

The effect of dibutyryl cyclic AMP on the membrane potential of ganglion cells was also examined in the presence of theophylline which is known to be a phosphodiesterase inhibitor. Ganglion cells were never hyperpolarized but, again, slightly depolarized (less than 1 mV) by dibutyryl cyclic AMP (1 mM) in the presence of theophylline (2 mM). An example of this experiment is shown in Figure 1, a3 and b3.

The effect of dibutyryl cyclic AMP on the resting membrane potential of ganglion cells was also studied by recording the intracellular potential of individual ganglion cells. Detectable hyperpolarization was never observed in the presence of dibutyryl cyclic AMP (1 mM) in the absence or presence of theophylline (2 mM), a finding which is in agreement with the recent report by Kuba & Nishi (1976). However, it should be noted here that adrenaline-induced hyperpolarizations have not been recorded intracellularly in this particular preparation (Nakamura & Koketsu, 1972).

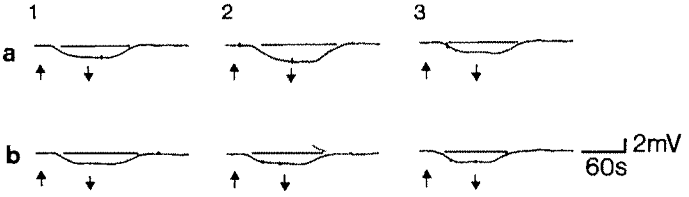

**Figure 2** The effect of (a) adrenaline (0.3 mM) and (b) dibutyryl cyclic AMP (1 mM) on the K$^+$-activated hyperpolarization recorded from a sympathetic ganglion by the sucrose-gap method. (a and b) Records 1, and 2 were before and 20 min after application of drugs, respectively, and records 3 were 30 min after withdrawal of these drugs. These K$^+$-activated hyperpolarizations were produced by changing the perfusate from the K$^+$-free solution to the Ringer solution; the duration of applications of the Ringer solution is indicated by arrows. Note an augmentation of the K$^+$-activated hyperpolarization in the record a2, and its absence in record b2.

### *Effect of dibutyryl cyclic AMP on the K$^+$-activated hyperpolarization*

It has been suggested that the slow i.p.s.p. and also adrenaline-induced hyperpolarization might be generated by an activation of the electrogenic Na$^+$ pump (Koketsu & Nishi, 1967; Nishi & Koketsu, 1967; 1968b; Nakamura & Koketsu, 1972; Koketsu & Nakamura, 1975; 1976). In connection with this suggestion, it was demonstrated (Akasu & Koketsu, 1976a,b) that adrenaline was able to potentiate the K$^+$-activated hyperpolarization which was generated by an activation of the electrogenic Na$^+$ pump (Rang & Ritchie, 1968; see also Akasu, Shirasawa & Koketsu, 1975). If cyclic AMP mediates such an action of adrenaline, it is to be expected that the amplitude of the K$^+$-activated hyperpolarization would be augmented in the presence of dibutyryl cyclic AMP. Experimental procedures for recording the K$^+$-activated hyperpolarization from the preparations and for examining the effect of a drug on this hyperpolarization by use of the sucrose-gap method were essentially similar to those described elsewhere (Akasu *et al.*, 1975; Akasu & Koketsu, 1976b). The augmentation of the K$^+$-activated hyperpolarization of ganglion cells was first confirmed in the presence of adrenaline (0.3 mM), then dibutyryl cyclic AMP was added after the withdrawal of adrenaline. The K$^+$-activated hyperpolarization recorded 20 min after an application of dibutyryl cyclic AMP showed no significant changes, as shown in Figure 2.

### *Effects of theophylline on the adrenaline-induced hyperpolarization*

The adrenaline-induced hyperpolarizations of ganglion cells were recorded by use of the sucrose-gap method (cf. Nakamura & Koketsu, 1972). The effect of theophylline (2 mM) on these hyperpolarizations was examined. In 5 preparations, within approximately 15 min after addition of theophylline, the adrenaline-induced hyperpolarizations were never augmented. In 3 preparations they were depressed, as shown in record a3 of Figure 1.

### *Effects of theophylline on slow potentials*

The P- and LN-potentials could be recorded from nicotine-treated or curarized ganglia by applying preganglionic nerve stimulation (30 Hz for 4 s) (Koketsu & Nishi, 1967; Nishi & Koketsu, 1967; 1968b). The effect of theophylline on these potentials was examined by use of the sucrose-gap method. In 11 preparations, within approximately 15 min after an addition of theophylline (2 mM) to the perfusate, the amplitude of P-potential and the LN-potential were depressed. These actions of theophylline were completely reversible; the amplitude of the P-potential and the LN-potential returned to the control value within approximately 30 min after theophylline was withdrawn. An example of these experiments is shown in Figure 3. The depression of LN-potentials by theophylline varied depending on individual ganglia. Thus, when the depression was strong, the initial part of LN-potentials even reversed potential polarity, as seen in Figure 3c. Such a unique change in the response (P- and LN-potentials) appeared to be explained by the change in the slow e.p.s.p. (see Discussion section).

In bullfrog sympathetic ganglia in which nicotinic transmission is completely blocked in the presence of nicotine (0.24 mM), or (+)-Tc (0.14 mM), the slow e.p.s.p. can be recorded easily with an intracellular electrode inserted into a cell-body of the ganglion cell (Nishi & Koketsu, 1968a; Tosaka, Chichibu & Libet, 1968; Kuba & Koketsu, 1974; 1976). Although the slow i.p.s.p., which precedes the slow e.p.s.p., can be recorded by an intracellular microelectrode from some cells, particularly from C-type cells (Tosaka *et al.*, 1968), the size of recorded slow i.p.s.p. is always very small (less than 2 mV) compared with the slow e.p.s.p. (cf. Figure 4b).

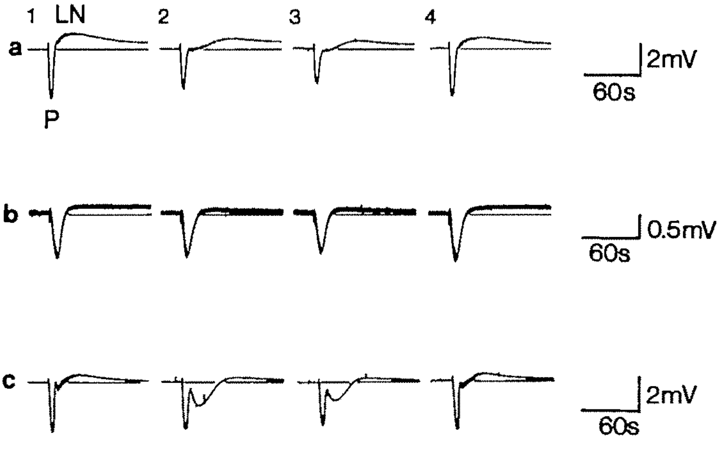

**Figure 3** The effect of theophylline (2 mM) on the P- and LN-potential recorded from the nicotine-treated (a and c) and curarized (b) sympathetic ganglia. Records 1 were control, records 2 and 3 were obtained 15 min and 30 min after application of theophylline, respectively, and records 4 were approximately 30 min after its withdrawal. Note the depression of P- and LN-potentials in (a) and (b), and a unique change of the potential pattern in (c) (see text).

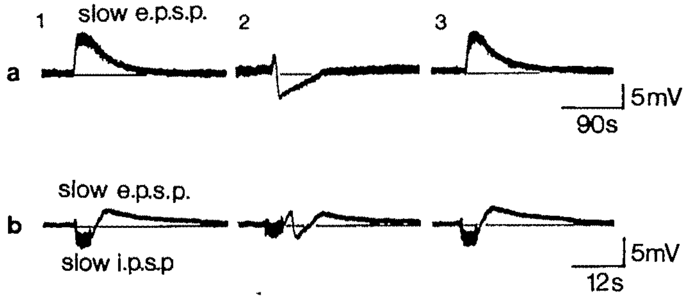

**Figure 4** Effects of theophylline (2 mM) on the slow e.p.s.p. (a and b) and slow i p.s p. (b) recorded by intracellular microelectrodes from two different nicotine-treated sympathetic ganglion cells. These potential changes were evoked by preganglionic nerve stimulations (30 Hz for 4 s); the slow i.p.s.p which precedes the slow e.p.s p. is seen only in (b). (a and b) Records 1 and 2 were before and 15 min after application of theophylline, and records 3 were 30 min after its withdrawal. Note theophylline-induced hyperpolarizations triggered by the slow e.p.s.p. (a and b), and a depression of the slow i.p.s.p. (b) with theophylline.

When theophylline (2mM) was applied to nicotine-treated or curarized ganglia for more than approximately 10 min, the ganglion cell membrane was spontaneously and transiently hyperpolarized with a characteristic periodicity. These theophylline-induced hyperpolarizations were also observed in the absence of nicotine or (+)-Tc and were essentially similar to the caffeine-induced hyperpolarizations recently reported by Kuba & Nishi (1976). The slow e.p.s.p. recorded in the presence of theophylline was abolished by a transient large hyperpolarization. When the slow e.p.s.p. was generated by preganglionic stimulation, its initial depolarization triggered a large transient hyperpolarization which was similar to the spontaneously occurring hyperpolarization and ranged from 5 to 20 mV (Figure 4).

The size of the slow i.p.s.p. was never augmented but rather tended to be depressed in the presence of theophylline. These results were similar to those observed in the P-potential. An example of these results is shown in Figure 4b. These effects of theophylline on the ganglion cells were completely reversible; the hyperpolarization triggered by the slow e.p.s.p. and also the other theophylline-induced hyperpolarizations disappeared within approximately 30 min after theophylline was withdrawn. It should be noted that these theophylline-induced hyperpolarizations were recorded, as in the case of caffeine-induced hyperpolarizations (Kuba & Nishi, 1976), from a limited proportion of cells.

## Discussion

In the present experiments, dibutyryl cyclic AMP caused no hyperpolarization in the bullfrog sympathetic ganglia, in which the adrenaline-induced hyperpolarization was actually observed. Furthermore, theophylline did not potentiate but rather tended to depress the P-potentials in curarized or nicotine-treated ganglia, and depressed the adrenaline-induced hyperpolarization. None of these results support the hypothesis (McAfee & Greengard, 1972) that the P-potential (slow i.p.s.p.) as well as the adrenaline-induced hyperpolarization of sympathetic ganglia are produced by an increase of cyclic AMP in ganglion cells. Discrepancies between the results obtained from mammalian (McAfee & Greengard, 1972) and present preparations are difficult to explain satisfactorily at present, although they might be simply due to a difference between species.

A facilitating effect of dibutyryl cyclic AMP on the $K^+$-activated hyperpolarization was not observed in our experiments on the sympathetic ganglion cells in which the facilitating effect of adrenaline on the $K^+$-activated hyperpolarizations had been confirmed. This suggests that the action of adrenaline on the $K^+$-activated hyperpolarization may not be mediated by cyclic AMP. It should be noted here that the $K^+$-activated hyperpolarization of ganglion cells is considerably augmented in the presence of ATP (Akasu, 1976).

Although methylxanthines (e.g. caffeine, theophylline) are known to inhibit the enzyme activity of phosphodiesterase which catalyzes cyclic AMP, it is also known that these drugs release $Ca^{2+}$ from sarcoplasmic reticulum into the myoplasm (Weber & Herz, 1968) and increase $Ca^{2+}$ permeability of the myoplasmic membrane (Bianchi, 1961). Kuba & Nishi (1976) suggested that the caffeine-induced hyperpolarizations were the result of an increased conductance to $K^+$ which was probably caused by a rise in the intracellular $Ca^{2+}$ concentration. In this way, the depression of LN-potential by theophylline was due to the generation of theophylline-induced hyperpolarizations. Indeed, the slow e.p.s.p. was abolished when these theophylline-induced hyperpolarizations were triggered by slow e.p.s.p., during which the intracellular $Ca^{2+}$ concentration might be increased (Kuba & Koketsu, 1974; 1976). Since these hyperpolarizations are generated only from limited numbers of ganglion cells producing slow e.p.s.p., the degree of depression of the LN-potential would vary depending on individual ganglia. The pattern of LN-potential shown in Figure 3 appeared to be due to a large number of cells producing these hyperpolarizations triggered by slow e.p.s.p.

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# THE INTERACTION OF SOME *BIS*-ARYLHYDROXYSULPHONIC ACIDS WITH A SITE OF KNOWN STRUCTURE IN HUMAN HAEMOGLOBIN

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1 Two *bis*-arylhydroxysulphonic acids were previously designed to interact with the known molecular configuration of the 2,3-diphosphoglycerate (DPG) receptor-site of human haemoglobin. These compounds liberate oxygen from the haemoglobin similarly to DPG.

2 Solutions of haemoglobin have now been observed under physiological conditions by nuclear magnetic resonance (n.m.r.) in the presence of DPG and of the compounds.

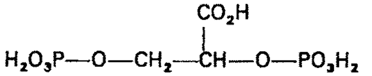
3 Two peaks in the n.m.r. spectrum of haemoglobin are shifted when DPG is added to the solution.

4 The same two peaks in the spectrum are affected by the compounds.

5 The observations are compatible with the predicted interaction between the compounds and the haemoglobin receptor site.

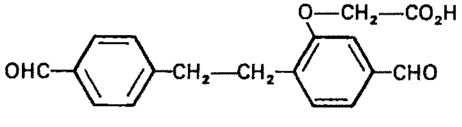
## Introduction

There is a site of known molecular structure in the human haemoglobin molecule, at which 2,3-diphosphoglycerate (DPG) interacts and promotes the liberation of oxygen (Arnone, 1972). This site (Figure 1) has been used as a model pharmacological receptor by Beddell, Goodford, Norrington, Wilkinson & Wootton (1976), who designed and synthesized three novel compounds which should also interact with it:

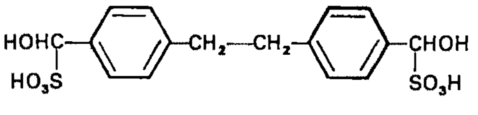


$$H_2O_3P{-}O{-}CH_2{-}\overset{CO_2H}{\overset{|}{C}}H{-}O{-}PO_3H_2$$

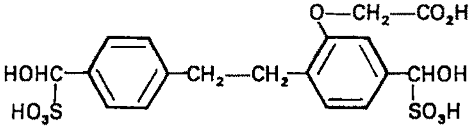
(DPG)



(I)



(II)



(III)

They predicted that these compounds, like DPG itself, should promote the liberation of oxygen from haemoglobin, and this was found to be the case. To this extent at least their approach was therefore successful but no direct evidence confirmed that the compounds were in fact reacting with the DPG site as intended.

The present paper describes some observations by nuclear magnetic resonance (n.m.r.) on the interaction of two of the compounds (the *bis*-arylhydroxysulphonic acids II and III) with human haemoglobin under physiological conditions similar to those used by the original authors.

## Methods

### *Preparation of solutions*

Human haemoglobin was prepared from freshly drawn blood as described by Brown, Halsey & Richards (1976) except that 5 mmol dm$^{-3}$ sodium metabisulphite was added to the final solution as described by Beddell *et al.* (1976). The haemoglobin was deoxygenated in a IL 237 Tonometer which allowed equilibration with oxygen-free nitrogen. pH was measured *in situ*, and was adjusted by admixture with high pH or low pH solutions of haemoglobin. Temperature was controlled by a water jacket thermostatted to 37°C. A permanent over pressure of

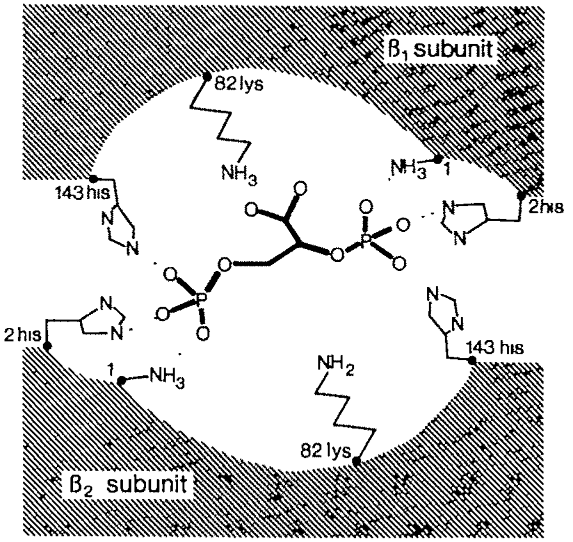


**Figure 1** A schematic representation of the 2,3-di-phosphoglycerate (DPG) receptor site in the human haemoglobin molecule, with DPG bound as observed by Arnone (1972).

nitrogen minimized contamination of the gas phase from the atmosphere.

An exit tube acted as gas outlet and an inlet tube could be connected either to a haemoglobin reservoir for pH adjustment, or to a 3-way tap whose second port held a 1 ml syringe for injection of solutions of the compounds. These were always dissolved in the same phosphate buffer as that used for the haemoglobin preparation. The third port of the tap held a large syringe of nitrogen gas which was used to force the solutions down the tube and into the equilibration chamber. Samples could be withdrawn from the equilibration chamber by pushing the mouth of the exit tube below the surface of the liquid which was then forced through the exit tube by the nitrogen pressure and into an n.m.r. sample tube which had been thoroughly flushed with nitrogen and could then be sealed.

### *Nuclear magnetic resonance*

The samples were studied on a Brucker 270 MHz Fourier Transform n.m.r. Spectrometer to obtain their proton spectra. The data were accumulated by the spin echo method of Campbell, Dobson, Williams & Wright (1975) when a $90°$-$\tau$-$180°$-$\tau$ sequence of pulses and delays were applied. This procedure allows for the collection of an echo signal after the second delay, $\tau$. No information appears in the echo from peaks whose relaxation times are short enough for their signal to have decayed within the time $2\tau$. It was found that if $\tau = 25$ ms, the only peaks remaining in the aromatic region of the spectrum were those from the C2 and C4 protons of surface histidine residues. All the spectra presented here are of this type, and peaks are numbered with Roman numerals according to the nomenclature of Brown *et al.* (1976).

The change in the n.m.r. signal which occurs when an added compound interacts with a resonant atom at a binding site is determined both by the change in chemical shift which occurs on binding, $\Delta\omega$, and by the lifetime t in the bound state. When $t.\Delta\omega \ll 1$, then the compound does not remain bound for long enough for the nuclei at the binding site to distinguish a clearly defined environment from either the bound or the unbound state. Instead, a weighted average is observed dependent on the relative proportions present in each state. This is the fast exchange case and is characterized by a single sharp signal whose position will move between the two extremes. As $t.\Delta\omega$ increases, so the line width increases reaching a maximum at $t.\Delta\omega \simeq 1$. Above this the bound and unbound states begin to become discernible as separate environments and the broad line separates into two lines. In the limit of slow exchange $t.\Delta\omega \gg 1$ the bound and unbound states both behave as completely separate entities.

The value of $\Delta\omega$ can vary greatly with the nature of the interaction. In the case of DPG the perturbation of the histidine ring protonation by the proximity of a negatively charged group (phosphate) is being observed. The effect of the perturbation is to stabilize the positively charged protonated form of the interacting ring in relation to the neutral form. The ring systems in these two forms differ considerably in the magnitude of the field induced in them by the applied magnetic field and hence in the chemical shift of the protons joined to the ring (i.e., the C2 and C4 protons). As a result such protons can be used to monitor the state of the rings whose nitrogen atoms will titrate exhibiting characteristic pKs which one would expect to increase in the presence of an interacting DPG molecule. Such an interpretation assumes that both the DPG and the protonating proton are in fast exchange with the histidine, although this situation could alter at lower pH values when the charges on the histidines and on the DPG change.

## Results

Figure 2a shows the aromatic region of the nuclear magnetic resonance spectrum from a 2 mmol dm$^{-3}$ aqueous solution of human deoxyhaemoglobin at pH 7.7. The spectrum is well resolved under our experimental conditions, and a number of sharp resonances from the protein can be identified and numbered. At this particular pH, however, peaks II and IV overlap, and so do peaks VIII and IX, and it

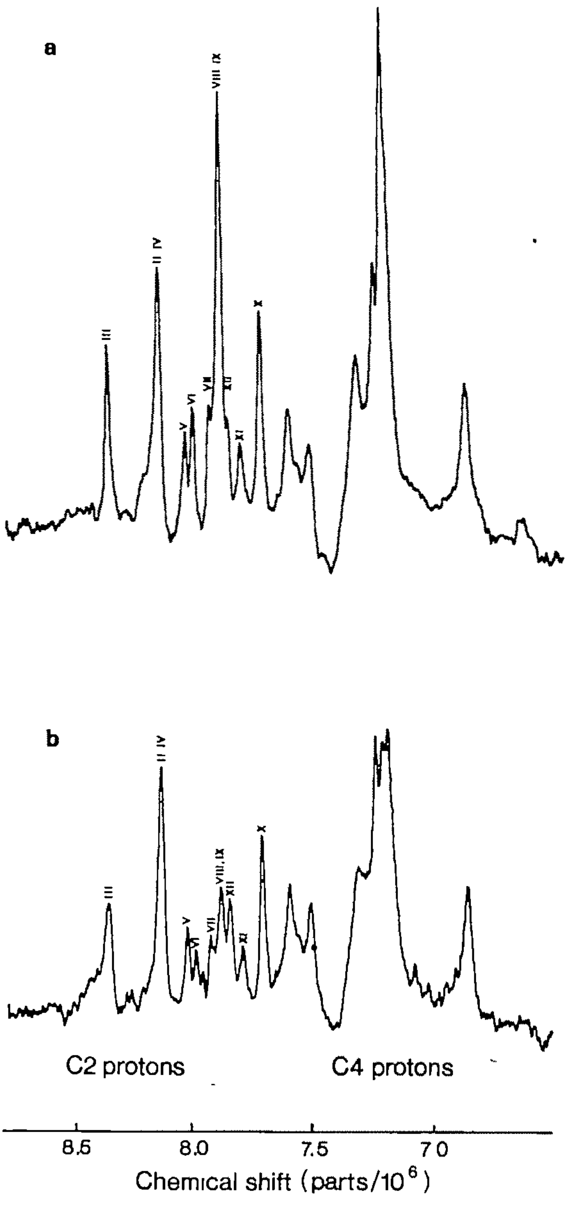


**Figure 2** (a) The aromatic region of the n.m.r. spectrum from 2 mmol dm$^{-3}$ human deoxyhaemoglobin at pH 7.7. All chemical shifts are measured relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate. (b) The same deoxyhaemoglobin sample in the presence of 1 mmol dm$^{-3}$ compound III. This compound has aromatic peaks in the region 7–7.6 parts/10$^6$ and would not interfere with the C2 spectrum. Note the collapse of peaks VIII and IX in the presence of the compound.

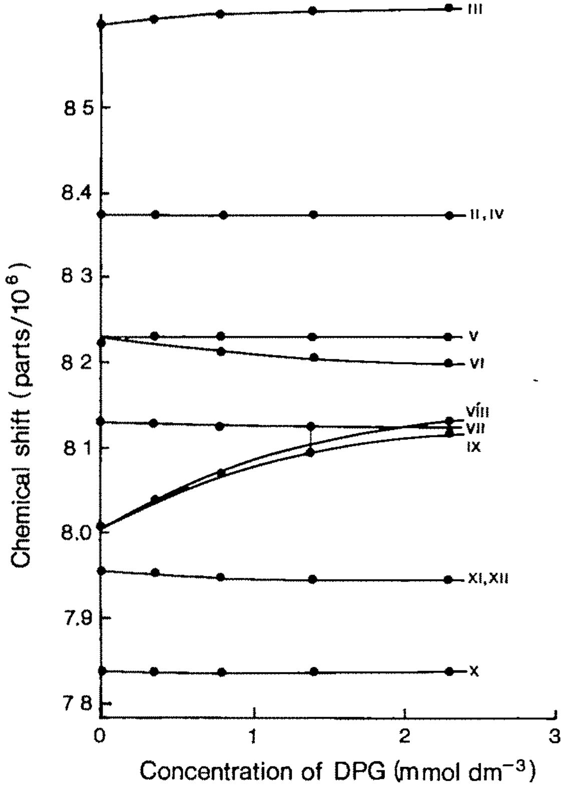


**Figure 3** The chemical shifts of the C2 protons of histidine residues in 2 mmol dm$^{-3}$ human deoxyhaemoglobin at pH 7.3 plotted as a function of added 2,3-diphosphoglycerate (DPG) concentration. Correction has been made, from pH titration curves, for the small changes in chemical shift due to small fluctuations in pH in different samples. Peak numbering corresponds with the Roman numerals in Figure 2 (see text). Note the shift of peaks VIII and IX in the presence of DPG.

would be necessary to make observations at different pHs in order to resolve these resonances.

*Control observations on 2,3-diphosphoglycerate*

When increasing concentrations of DPG are added to the solution the twin peaks VIII and IX are progressively shifted to a much greater extent than any other resonances. This is shown in Figure 3 where the position of each peak in the n.m.r. spectrum is plotted as a function of DPG concentration. Peaks VIII and IX move downfield corresponding to an increase in pK which is in line with the above expectations.

These DPG observations were made at a physiological pH of 7.3, but below pH 7.0 the situation changes and the peaks broaden in acid solutions. The effects have already been studied in some detail (Brown & Campbell, personal communication), and pH titration curves have been determined. The relevant point in the present context is that DPG is always observed to affect peaks VIII and IX most strongly (Figure 3). On the basis of the X-ray crystallographic evidence (Arnone, 1972) for the DPG-haemoglobin interaction, these two peaks are therefore tentatively assigned to the C2 protons of histidines $\beta 2$ and $\beta 143$. A similar effect is observed for the C4 protons (not shown here).

### *Observations on the compounds*

Examination of the effects of compounds II and III revealed a collapse of the same two resonances at all pH values observed. As a result the peaks apparently vanish from the n.m.r. spectrum. Figure 2b illustrates this effect for compound III which is the more effective of the two. It is an unfortunate coincidence that peaks VIII and IX cannot be distinguished because they super-impose in this physiological range of pH (Figure 2).

These results strongly suggest that both DPG and compounds II and III interact at the same binding site, even though the assignment to histidines $\beta 2$ and $\beta 143$ may not be established unequivocally. For the interaction of DPG it appears that the exchange is fast ($t.\Delta\omega \ll 1$), and the shift is of the order of 40 Hz corresponding to a pK increase of a little over 0.5 for peaks VIII and IX in the bound state. In the case of compounds II and III the exchange is slower ($t.\Delta\omega \gg 1$) and the collapse of peaks VIII and IX in the unbound haemoglobin is not matched by the emergence of a signal from the bound state, so that an estimate for $\Delta\omega_{cpd}$ cannot be made.

Apart from the main effects observed for peaks VIII and IX there are a number of minor effects. It will be observed in Figure 3 that DPG causes a slight downfield shift of peak III which is known to be histidine $\beta 146$ (Kilmartin, Breen, Roberts & Ho, 1973), and a slight upfield shift of peak VI. It is quite possible that peak VI could be histidine $\beta 97$ since histidines $\beta 2$, $\beta 97$, $\beta 143$ and $\beta 146$ all lie quite near each other. Compounds II and III are also observed (Figure 2b) to affect these resonances. Peak III is broadened and peak VI collapses, and although the effect is very much less marked than for peaks VIII and IX, this observation again supports the prediction of Beddell *et al.* (1976) that compounds II and III should interact at the DPG site. However, there is one difference observed between the compounds and DPG and that is an upfield shift of peak IV. This secondary difference from DPG may be due to more subtle conformational changes induced, perhaps, by covalent binding of the compounds.

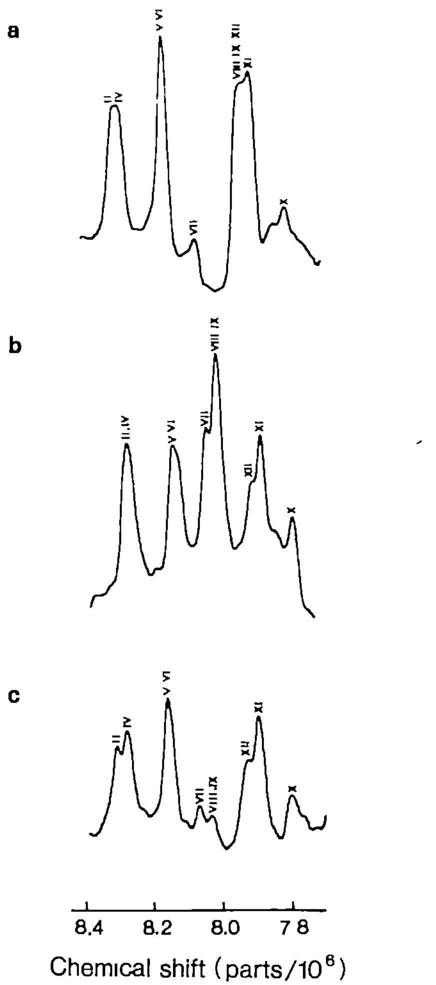


**Figure 4** (a) The histidine C2 proton resonances from 2 mmol dm$^{-3}$ deoxyhaemoglobin at pH 7.4. (b) The same sample in the presence of 1 mmol dm$^{-3}$ 2,3-diphosphoglycerate (DPG) which shifts peaks VIII and IX away from peaks XII and XI, and onto the side of peak VII. (c) The same sample to which a further 1.5 mmol dm$^{-3}$ compound III has been added. The shifted peaks VIII and IX collapse as would be expected if both DPG and compound III had a common binding site.

### *Observations on 2,3-diphosphoglycerate and the compounds together*

Although the previous observations are strongly indicative of a common binding site for DPG and the bibenzyl compounds, they are still open to the objection that different but coincidentally superimposed resonances might have been affected by the different agents. To deal with this criticism DPG was first used to shift the double peak (VIII + IX) into another position before it was collapsed by one of the compounds. Figure 4a is the control spectrum and Figure 4b shows a downfield shift of the double peak (VIII + IX) in the presence of DPG, exposing peak XII and covering peak VII. On the further addition of compound III shown in Figure 4c, the collapse of this double peak in its new position is observed. Because of the large difference in rates of exchange for the two compounds, one does not observe any change in the shift caused by DPG. Instead one still observes the fast exchange of DPG with the few remaining binding sites which are not blocked by compound III. Thus the shifted resonances simply collapse, which would be expected under these conditions if both DPG and compound III have a common binding site. Furthermore the residual peaks XI and XII are unaffected by the addition of compound III to the mixture of haemoglobin and DPG, showing that the effect on the region of the spectrum between 7.8 and 8.1 parts/$10^6$ is confined exclusively to the resonances which are influenced by DPG itself. Similar observations were also made with compound II.

## Discussion

The results illustrated here appear to be entirely consistent with the mode of binding already postulated by Beddell *et al.* (1976) for compounds II and III. The primary effect of these compounds on the n.m.r. spectrum is to collapse the same two resonances which are shifted by DPG. Their long lifetimes in the bound state relative to DPG are exactly what would be anticipated from compounds such as these which would be expected to bind in a covalent manner at the predicted site. The effect of compound III is more pronounced than compound II in terms of its ability to collapse peaks VIII and IX, which suggests weaker binding by compound II and is in line with the relative abilities of the compounds to liberate oxygen from haemoglobin as described in the original paper.

The differences observed between compounds II and III and DPG are at present a matter of pure speculation. It would be surprising if no small differences between the two classes of compounds were observed since DPG is basically hydrogen-bonded to the site, whereas the compounds II and III may form covalent links to the terminal amino groups of the $\beta$-chains. However, the prime objective of this study was to identify the site of binding, and the present observations are compatible with the predictions of Beddell *et al.* (1976) for compounds II and III, although they do not preclude the presence of other binding sites elsewhere on the protein and throw no light on the binding of compound I. Subject to these limitations, however, they support the suggestion that it may be possible to design drugs to interact with receptor sites of known molecular structure and produce predefined biological effects.

We are grateful to Dr Ian Campbell for valuable discussions, and to Mr Peter Bateman and Miss Elizabeth Rahr for their help in preparing the samples.

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# EVIDENCE FOR DOPAMINE DEAMINATION BY BOTH TYPE A AND TYPE B MONOAMINE OXIDASE IN RAT BRAIN *in vivo* AND FOR THE DEGREE OF INHIBITION OF ENZYME NECESSARY FOR INCREASED FUNCTIONAL ACTIVITY OF DOPAMINE AND 5-HYDROXYTRYPTAMINE

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1 Tranylcypromine (20 mg/kg) administration to rats totally inhibited brain monoamine oxidase (MAO) oxidation of 5-hydroxytryptamine (5-HT), phenylethylamine and dopamine as measured *in vitro*. When L-3,4-dihydroxyphenylalanine (L-DOPA) (50 mg/kg) was given 30 min after the tranylcypromine, brain dopamine and noradrenaline concentrations rose markedly and the rats displayed characteristic behavioural changes and locomotor activity.

2 Clorgyline (5 mg/kg) administration inhibited 5-HT oxidation by almost 100% but phenylethylamine by only 29% while (−)-deprenil (5 mg/kg) injection almost totally inhibited phenylethylamine oxidation and inhibited 5-HT metabolism by only 31%. Administration of L-DOPA after pretreatment with either drug did not alter brain dopamine or noradrenaline concentrations and the animals did not display any behavioural changes.

3 Administration of clorgyline plus (−)-deprenil (5 mg/kg of each) almost totally inhibited oxidation of both phenylethylamine and 5-HT; there was a large rise of brain dopamine and noradrenaline concentrations and the animals displayed the behavioural changes observed when tranylcypromine and L-DOPA had been given.

4 The effects of tranylcypromine (20 mg/kg) on brain 5-HT, dopamine and noradrenaline concentrations up to 48 h after injection were recorded. Brain 5-HT concentrations were considerably elevated for 18 h after injection and then fell steadily. In contrast, brain dopamine concentrations rose slightly and remained at this level for 48 h while noradrenaline levels doubled and also remained at this level for 48 hours.

5 When L-tryptophan (50 mg/kg) was given at various times after tranylcypromine the characteristic hyperactivity syndrome appeared at 12 h but not 18 h after tranylcypromine and a further rise in brain 5-HT was only observed at 12 hours. When L-DOPA (50 mg/kg) was given at various times after tranylcypromine a further large rise in brain dopamine and noradrenaline occurred at 12 h but not at 18 h and all the behavioural changes were observed only at 12 hours.

6 Measurement of MAO activity at the above times after tranylcypromine showed that the half-life of recovery of the enzyme activity with 5-HT and dopamine as substrates was 4.5 days and 8.5 days with phenylethylamine as substrate. Inhibition of MAO oxidation of dopamine and 5-HT was approximately 85%, 18 h after tranylcypromine injection.

7 It is concluded from both the studies with clorgyline and deprenil and the recovery of MAO activity after tranylcypromine, that dopamine is metabolized by both Type A and Type B MAO *in vivo* and that it is only when both forms are almost totally inhibited that there is an increase in dopamine and 5-HT functional activity, as judged by the appearance of the hyperactivity syndromes.

## Introduction

In a previous paper we demonstrated that while *in vitro* it could be shown that 5-hydroxytryptamine (5-HT) was metabolized by the Type A form of monoamine oxidase (MAO-A), nevertheless when this form was inhibited *in vivo* by clorgyline, Type B monoamine oxidase (MAO-B) continued to metabolize the amine (Green & Youdim, 1975). Recently Ekstedt (1976) has shown that *in vitro* 5-HT is metabolized by MAO-B in liver mitochondria when MAO-A is inhibited by clorgyline.

Previous *in vitro* experiments have suggested that dopamine is metabolized by both forms of the enzyme (see Houslay, Tipton & Youdim, 1976) and we have now investigated whether this is true *in vivo* in the brain. To do this, both biochemical measurements and a behavioural model have been used in an analagous way to the previous study on 5-HT. The behaviour studied was that of the locomotor activity which follows administration of an MAO inhibitor and L-3,4-dihydroxyphenylalanine (L-DOPA) and which has been shown to be the result of increasing dopamine concentrations in the brain (Everett, Wiegland & Rinaldi, 1963; Everett, 1966; Green & Kelly, 1976).

Results indicate that both MAO-A and MAO-B must be inhibited by about 85% to increase dopamine functional activity. This observation led to the second part of this study, since our previous investigation (Green & Youdim, 1975) also indicated that this degree of inhibition of both enzyme types was necessary in order to increase 5-HT functional activity, following subsequent tryptophan administration.

We have now therefore also examined the length of time after administration to rats of tranylcypromine that an increase in functional activity of 5-HT or dopamine (as indicated by the appearance of the behavioural changes following respectively L-tryptophan and L-DOPA) can be demonstrated. The rise in brain 5-HT and dopamine at this time has also been measured. Results have been compared to the degree of inhibition of MAO seen at various times after tranylcypromine injection. Results again indicate the importance of inhibiting MAO by about 85% in order to increase amine functional activity as judged by the hyperactivity syndrome.

## Methods

Male Sprague-Dawley rats (150–200 g) (Anglia Laboratory Animals, Alconbury, Huntingdon) were used in all experiments.

### *Deprenil and clorgyline experiments*

Tranylcypromine (Smith, Kline & French, Ltd.), clorgyline (May & Baker, Ltd.) or (—)-deprenil were dissolved in 0.9% w/v NaCl solution (saline) and injected intraperitoneally to two groups of 3 rats. After 30 min L-DOPA (50 mg/kg) suspended in saline containing 1% carboxymethylcellulose was given to one group and saline to the other group. After a further 90 min the rats were killed, the brains removed and divided along the mid-line. One half was homogenized in 0.32 M sucrose and MAO activity towards the substrates [1-$^{14}$C]-dopamine, [1-$^{14}$C]-5-hydroxytryptamine (both Radiochemical Centre, Amersham) and [1-$^{14}$C]-phenylethylamine (NEN Chemicals GmbH) measured by the method of Tipton & Youdim (1976). Protein was measured by the procedure of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard. Enzyme activity was calculated as nmol of deaminated product formed/mg protein per 30 min incubation and results expressed as the mean ± s.e. mean of the percentage inhibition compared to saline-injected controls. The other half of the brain was homogenized in acidified butanol, and noradrenaline and dopamine measured as described by Chang (1964).

Activity was measured on both groups of 3 animals for 120 min after administration of the MAO inhibitor using Animex activity meters (sensitivity and tuning 30 μA) as described elsewhere (Grahame-Smith, 1971; Green & Grahame-Smith, 1974). Behavioural changes following the L-DOPA administration were exactly as described by Green & Kelly (1976).

### *Time course after tranylcypromine experiments*

Tranylcypromine was dissolved in saline and injected intraperitoneally. After various times the animals were injected with either L-DOPA (50 mg/kg) suspended in saline containing 1% carboxymethylcellulose or L-tryptophan (50 mg/kg) dissolved in saline. After 60 min the rats were killed, the brains removed and divided along the mid-line. After L-DOPA injection, dopamine and noradrenaline were measured as described above and after L-tryptophan injection, 5-HT was measured by the method of Curzon & Green (1970). The other half of the brain was homogenized in 0.32 M sucrose and MAO activity measured as described above.

Activity was measured in groups of 3 animals for 60 min after amino acid administration by means of Animex activity meters as outlined above. Behavioural changes are described in the text.

## Results

### *Effect of L-DOPA on rat brain dopamine and noradrenaline concentrations and monoamine oxidase activity*

Rats were injected with saline and 30 min later given L-DOPA (50 mg/kg). After a further 90 min they were killed and dopamine, noradrenaline and MAO activity measured. L-DOPA did not alter MAO activity and caused only a small rise in noradrenaline and dopamine (Table 1).

### *Effect of tranylcypromine, clorgyline or (—)-deprenil administration on the locomotor activity following subsequent L-DOPA administration*

Tranylcypromine (20 mg/kg) administration 30 min before L-DOPA (50 mg/kg) resulted in the characteristic behavioural changes and locomotor activity previously described (Everett *et al.*, 1963; Green & Kelly, 1976; Figure 1). When clorgyline (5 mg/kg) or (—)-deprenil (5 mg/kg) was given in place of the tranylcypromine no significant increase in locomotor activity was observed (Figure 1). Even a dose of 10 mg/kg of either of these inhibitors before L-DOPA did not result in increased locomotor activity. However, administration of both inhibitors (clorgyline, 5 mg/kg plus (—)-deprenil, 5 mg/kg) did result in the behavioural changes when L-DOPA was given 30 min later (Figure 1). Without the second L-DOPA injection there was no hyperactivity following any of the inhibitors in agreement with previous observations (Green & Youdim, 1975).

### *Effect of tranylcypromine, clorgyline or (—)-deprenil on brain dopamine and noradrenaline concentrations and monoamine oxidase activity*

When tranylcypromine (20 mg/kg) was given before the L-DOPA there was a large rise in brain dopamine and noradrenaline concentrations and MAO activity was totally inhibited with all substrates tested, 5-HT being used as an *in vitro* index of MAO-A activity and phenylethylamine for MAO-B (Table 1). In contrast neither clorgyline nor (—)-deprenil alone (at a dose of 5 mg/kg) produced as large a rise of dopamine and noradrenaline on subsequent L-DOPA injection, as that seen when tranylcypromine was given. When both clorgyline and deprenil were given together (5 mg/kg of each) brain dopamine and noradrenaline rose to the values seen when tranylcypromine (20 mg/kg) had been given. Almost total inhibition of

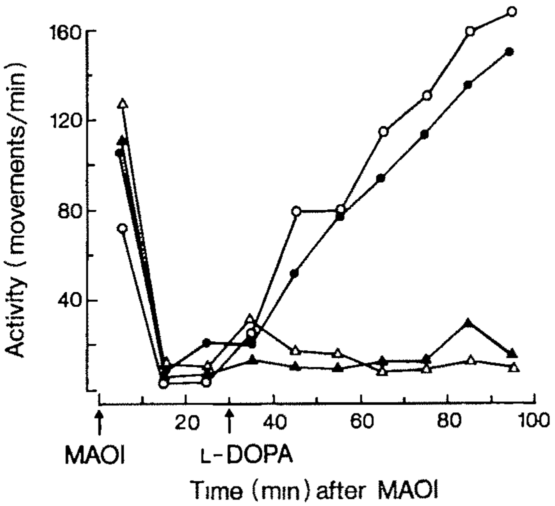

**Figure 1** Effect of L-DOPA administration to rats pretreated with clorgyline, deprenil or tranylcypromine. Groups of rats were injected with a monoamine oxidase inhibitor (MAOI), either clorgyline 5 mg/kg (▲), (—)-deprenil 5 mg/kg (△), clorgyline (5 mg/kg) plus (—)-deprenil (5 mg/kg) (●) or tranylcypromine (○). Thirty min later all groups were given L-DOPA (50 mg/kg). Figure shows the result of a typical experiment showing the change in locomotor activity (measured in movements/min) in the different groups of rats. Rats injected with either L-DOPA alone or tranylcypromine, clorgyline or (—)-deprenil (without subsequent L-DOPA injection) do not display increased locomotor activity.

**Table 1** Effect of monoamine oxidase inhibitors with or without L-DOPA (50 mg/kg) injection 30 min later on brain noradrenaline and dopamine concentrations and monoamine oxidase (MAO) activity towards 5-hydroxytryptamine (5-HT), dopamine and phenylethylamine 90 min after initial injection

| *Injected* | | *Brain catecholamine concentration (μg catecholamine/g brain (wet wt.))* | | *% Inhibition of MAO activity towards substrates* | | |
|---|---|---|---|---|---|---|
| *0 min* | *30 min* | *Noradrenaline* | *Dopamine* | *5-HT* | *Dopamine* | *Phenylethylamine* |
| Saline | Saline | 0.23 ± 0.02 (7) | 1.65 ± 0.10 (7) | —† | —† | —† |
| Saline | L-DOPA | 0.23 ± 0.02 (6) | 1.82 ± 0.12 (6) | 0 | 0 | 0 |
| Clorgyline (5 mg/kg) | L-DOPA | 0.33 ± 0.05 (6) | 1.66 ± 0.28 (6) | 98 ± 1 (12) | 87 ± 5 (12) | 29 ± 10 (3) |
| Deprenil (5 mg/kg) | L-DOPA | 0.26 ± 0.03 (6) | 1.72 ± 0.05 (6) | 31 ± 4 (12) | 43 ± 4 (12) | 91 ± 4 (3) |
| Clorgyline + deprenil (5 mg/kg of each) | L-DOPA | 0.44 ± 0.02 (4) | 6.05 ± 0.04 (4) | 92 ± 1 (7) | 89 ± 1 (7) | 99 ± 3 (7) |
| Tranylcypromine (20 mg/kg) | L-DOPA | 0.47 ± 0.02 (6) | 8.80 ± 0.85 (6) | 100 | 100 | 100 |

Brain noradrenaline and dopamine concentrations shown 90 min after MAO inhibitor injection, when L-DOPA had been given 30 min later. Brain MAO activity shown at same time. Results expressed as mean ± s.e. mean with number of determinations in brackets. Absolute MAO activities (in nmol deaminated product formed per mg protein/30 min incubation); dopamine: 59.2 ± 2.5; 5-HT: 75.6 ± 5.1; phenylethylamine: 51.5 ± 1.3 (all 6 determinations). Some MAO inhibition results taken from Green & Youdim (1975).

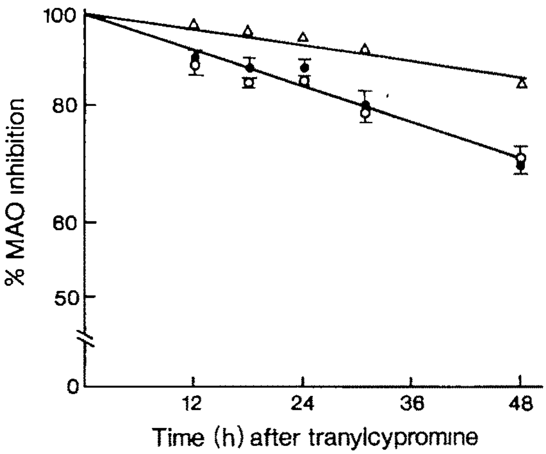

**Figure 2** Recovery of monoamine oxidase (MAO) activity, following injection of tranylcypromine. Rats were injected with tranylcypromine (20 mg/kg) and after various times they were killed and MAO activity measured with phenylethylamine (△), dopamine (○) and 5-hydroxytryptamine (●) as substrates. The half-life of reappearance of MAO activity was calculated and is reported in the text. Points show mean and bars ± s.e. mean of determinations in 6 rats. Where bars are not shown the s.e. mean was smaller than the point.

both forms of the enzyme was seen only when both inhibitors had been given (Table 1).

### *Return of monoamine oxidase activity following tranylcypromine administration*

Rats were injected intraperitoneally with tranylcypromine (20 mg/kg). At various times after the injection they were killed and MAO activity to 5-HT, dopamine and phenylethylamine measured. Results shown in Figure 2 demonstrate that the recovery of MAO activity towards 5-HT and dopamine was the same, with a half-life of 4.5 days, whereas the half-life with the substrate phenylethylamine was 8.5 days.

### *Brain 5-hydroxytryptamine, dopamine and noradrenaline at various times after tranylcypromine injection*

Following tranylcypromine (20 mg/kg) injection, brain 5-HT increased considerably being around 1 μg 5-HT/g (wet wt.) at both 12 and 18 h; after this time it started to decrease (Table 1). Brain dopamine concentration on the other hand showed a relatively small increase which was seen up to 48 h after injection. Brain noradrenaline concentration increased and also remained elevated for 48 h (Table 2).

### *Behavioural changes and brain 5-hydroxytryptamine concentrations following L-tryptophan administration to rats previously given tranylcypromine*

Administration of L-tryptophan (50 mg/kg) to rats given tranylcypromine (20 mg/kg) 30 min previously results in a complex series of behavioural changes including hyperactivity (Grahame-Smith, 1971). These changes were seen in this study (Figure 3) and also when the L-tryptophan was given 12 h after the tranylcypromine, although at this time the hyperactivity syndrome was seen to occur immediately after tryptophan administration (Figure 3). The activity was quantitatively similar to that seen in the rats given tranylcypromine 0.5 h before the tryptophan, although it could not be seen in these animals until about 40 min after tryptophan.

**Table 2** Changes in brain 5-hydroxytryptamine (5-HT), dopamine and noradrenaline at various times after injection of tranylcypromine (20 mg/kg) and the concentration of these amines 60 min after L-tryptophan or L-DOPA administration

| *Time (h) after tranylcypromine (20 mg/kg)* | *Brain amine concentrations in μg amine/g brain (wet wt.)* | | | | | |
|---|---|---|---|---|---|---|
| | *5-hydroxytryptamine* | | *Dopamine* | | *Noradrenaline* | |
| *Injected* | *Saline* | *L-tryptophan* | *Saline* | *L-DOPA* | *Saline* | *L-DOPA* |
| 0.5 | 0.53 ± 0.04 (6) | 0.93 ± 0.07 (6) | 1.65 ± 0.08 (6) | 8.80 ± 0.85 (6) | 0.23 ± 0.02 (7) | 0.47 ± 0.02 (6) |
| 12 | 1.01 ± 0.04 (8) | 1.43 ± 0.03 (4) | 2.64 ± 0.22 (3) | 6.93 ± 1.44 (3) | 0.46 ± 0 01 (3) | 0.67 ± 0.10 (3) |
| 18 | 0.98 ± 0.05 (4) | 0.91 ± 0.08 (6) | 3.32 ± 0.36 (4) | 3.37 ± 0.51 (3) | 0.42 ± 0.06 (4) | 0 48 ± 0.08 (3) |
| 24 | 0.73 ± 0.01 (4) | 0.93 ± 0.08 (6) | 2.66 ± 0.22 (4) | 4.30 ± 0.87 (9) | 0.59 ± 0.02 (4) | 0.56 ± 0.05 (6) |
| 48 | 0.57 ± 0.02 (4) | 0.62 ± 0.04 (3) | 2 48 ± 0 31 (4) | 3 36 ± 0.39 (6) | 0.43 ± 0.06 (3) | 0 42 ± 0.03 (6) |
| Saline | 0.44 ± 0.03 (6) | 0.66 ± 0.03 (4) | 1.72 ± 0.09 (11) | 1.94 ± 0.03 (3) | 0.23 ± 0.01 (9) | 0.24 ± 0.03 (3) |

Rats were injected with tranylcypromine (20 mg/kg). At various times after this injection they were killed and brain 5-HT, dopamine and noradrenaline measured while other groups were injected with either L-tryptophan (50 mg/kg), when brain 5-HT was measured 60 min later, or L-DOPA (50 mg/kg), when brain dopamine and noradrenaline were measured 60 min later.

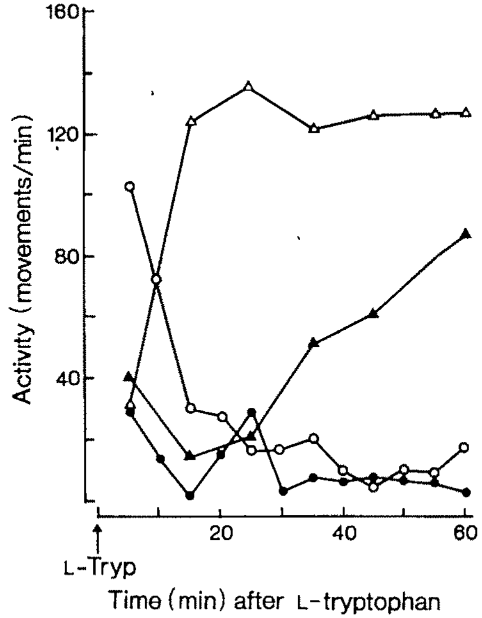

**Figure 3** Hyperactivity response of rats following L-tryptophan injection at various times after administration of tranylcypromine. Rats were injected with tranylcypromine (20 mg/kg). After 0.5 h (▲), 12 h (△), 18 h (●) and 24 h (○) they were given L-tryptophan (50 mg/kg) and activity measured over the next 60 min at which time they were killed and brain 5-hydroxytryptamine concentrations measured (Table 2). The 48 h result is not shown but was essentially the same as the 24 h result shown. No hyperactivity was observed in any group until after the L-tryptophan injection.

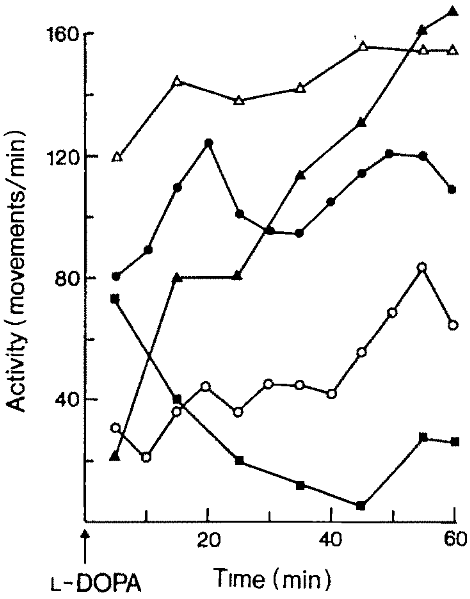

**Figure 4** Locomotor response of rats following L-DOPA injection at various times after administration of tranylcypromine. Rats were injected with tranylcypromine (20 mg/kg). After 0.5 h (▲), 12 h (△), 18 h (●), 24 h (○) and 48 h (■) they were given L-DOPA (50 mg/kg) and activity measured over the next 60 min at which time they were killed and brain dopamine and noradrenaline concentrations were measured (Table 2). No hyperactivity was observed in any group until after the L-DOPA injection.

The animals given tryptophan either 0.5 h or 12 h after tranylcypromine both showed a further rise in 5-HT above that produced by the MAO inhibitor.

In contrast, those animals given tryptophan 18 h, 24 h and 48 h after tranylcypromine showed neither a large 5-HT rise 60 min after L-tryptophan administration nor hyperactivity (Table 2 and Figure 3).

The hyperactivity syndrome was not seen in any group of animals until after the L-tryptophan administration.

### *Behavioural changes and brain dopamine and noradrenaline concentrations following L-DOPA administration to rats previously given tranylcypromine*

Administration of L-DOPA (50 mg/kg) to rats pretreated with tranylcypromine (20 mg/kg) either 0.5 h or 12 h earlier, results in a large brain dopamine rise (Table 2) and the appearance of all behavioural changes described by Green & Kelly (1976). These include irritability, squeaking, aggression, Straub tail, forepaw padding, headweaving, salivation, piloerection, rearing to other animals and locomotor activity. Eighteen and 24 h after tranylcypromine, L-DOPA administration resulted in a smaller dopamine rise than that seen after 12 hours. Furthermore, the behaviour changed. Locomotor activity occurred (although the effect was less by 24 h than at 18 h) but was not accompanied by many other of the behavioural features. There was little rearing, squeaking or aggression and headweaving, piloerection and forepaw padding were absent. The animals now looked much more as if they had been given methamphetamine at a low dose (1 mg/kg). At 18 h and 24 h only some of the animals displayed hyperactivity and this is probably due to the large variation at this time in brain dopamine levels from near normal to very elevated. No locomotor activity was observed when the rats were given L-DOPA 48 h after tranylcypromine and there was an even smaller brain dopamine rise, nor was it seen in any group until after the L-DOPA injection.

It was found that L-DOPA administration failed to alter significantly brain noradrenaline concentrations (Table 2). This is in accord with previous studies on the influence of L-DOPA administration on central noradrenaline concentrations (Everett & Borcherding, 1970; Green & Grahame-Smith, 1974).

## Discussion

The first part of this study clearly demonstrates that in the brain, dopamine is metabolized by both Type A and Type B MAO. Unless both forms of the enzyme are inhibited, L-DOPA administration neither produces the rise of brain dopamine nor the behavioural changes seen after administration of a non-specific inhibitor such as tranylcypromine. Similar observations have also been made by Squires (personal communication).

Previously Yang & Neff (1974) suggested that dopamine is metabolized by both Type A and Type B MAO on the basis of *in vitro* studies. While our *in vivo* work on hyperactivity and the rise in brain dopamine agrees with this observation, the estimation of MAO activity *in vitro* after rats have been treated *in vivo* with selective MAO inhibitors suggests that inhibition of dopamine metabolism is influenced much more by inhibition of Type A than Type B MAO (Table 1). This is also supported by the observation that the recovery of dopamine metabolizing activity *in vitro* following total inhibition by tranylcypromine injection exactly parallels the recovery of MAO activity towards 5-HT (a Type A substrate, Figure 2). Therefore, we suggest that dopamine may normally be metabolized by Type A MAO but that Type B enzyme can continue to metabolize the amine when the Type A enzyme is inhibited. This is exactly analagous to the situation with 5-HT in the brain (Green & Youdim, 1975).

Our figures on the half-life of the return of MAO activity following total inhibition agree with some of those obtained by Maître, Delini-Stula & Waldmeier (1976). For example they also found a half-life of the disappearance of inhibition of MAO of 8.5 days using phenylethylamine as a substrate, after giving either iproniazid or deprenil but not after tranylcypromine. When 5-HT was used as a substrate however they obtained a half-life of about 10 days which is considerably longer than that found here and again tranylcypromine was reported to show a different pattern from other inhibitors tested. However, like us, Maître *et al.* (1976) found a similar time course to 5-HT when dopamine was used as a substrate.

The results with clorgyline and (—)-deprenil showed that the large rise in brain dopamine and the behavioural changes occurred only when both forms of the enzyme were almost completely inhibited. This observation was taken one stage further by studying the recovery of MAO activity towards various substrates after total inhibition of the brain enzyme. It was found that when MAO inhibition dropped below about 85% (this occurs around 18 h after injection) L-tryptophan injection did not produce a large 5-HT rise nor did the animals display the hyperactivity syndrome. When L-DOPA was given at 18 h there was a smaller brain dopamine rise than that seen 12 h after tranylcypromine and some behavioural changes still occurred. However, the behavioural change was much less marked than that seen 12 h after injection and many of the behavioural components of the change were absent. These findings complement our previous suggestion on the basis of studies on 5-HT (Green & Youdim, 1975), that total MAO activity is present in the brain in large amounts and that it is only when both forms of the enzyme are inhibited by about 85% that there is an increase in the functional activity of 5-HT or dopamine.

This observation has implications in the use of MAO inhibitors in the treatment of psychiatric illness. First, because this degree of inhibition does not normally appear to be reached during administration of MAO inhibitors (Youdim, Collins, Sandler, Bevan-Jones, Pare & Nicholson, 1972) and second, because of the research being carried out on the feasibility of using selective monoamine oxidase inhibitors to increase preferentially the concentration of specified neurotransmitters.

We thank Smith, Kline and French for tranylcypromine, May and Baker for clorgyline and Professor J. Knoll (Budapest) for (—)-deprenil. We are also grateful to Dr R.F. Squires for sending his unpublished data and Professor D.G. Grahame-Smith for his helpful comments.

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# EFFECT OF TWO HEMICHOLINIUMS ON THE DISPOSITION AND DISTRIBUTION OF ENDOGENOUS FREE CHOLINE IN ANAESTHETIZED RABBITS

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1 The effects of hemicholinium no. 3 (HC-3) and its *p*-terphenyl analogue (TPHC-3) on the disposition and distribution of free choline were studied in rabbits under pentobarbitone anaesthesia. Free choline was determined by bioassay.

2 The respiration of animals given 0.35 μmol/kg of HC-3 was hardly affected; however, 4 out of 6 rabbits given the same dose of TPHC-3 exhibited varying degrees of respiratory impairment. All animals that received 2 injections (1 h apart) of either HC-3 (1.4 μmol/kg) or TPHC-3 (0.7 μmol/kg) developed respiratory difficulty about 1 h after the second injection.

3 The respiratory distress was accompanied by a 2 to 15-fold rise in plasma choline concentration. This rise has been attributed to hypoxia.

4 In experiments in which choline has been infused it was observed that HC-3 could impair the animal's ability to dispose of exogenous choline. In control rabbits neither HC-3 nor TPHC-3 produced changes in plasma choline concentrations unless the respiration was depressed.

5 Either HC-3 or TPHC-3 (both at 0.35 μmol/kg) significantly ($P < 0.05$) reduced the kidney choline concentration by 40% and 30% respectively; both hemicholiniums raised the lung choline concentration by about 35%. Only TPHC-3 caused a significant rise (40%) in liver choline. The choline concentrations in other tissues were unaffected by the hemicholiniums.

## Introduction

The hemicholiniums, especially hemicholinium-3 (HC-3) (Schueler, 1955) have been much used for the detection and study of cholinergic nerves. The basis for their pharmacological actions is generally believed to be an interference with the access of choline through cellular barriers to the sites where it is acetylated. They thus interrupt synaptic transmission when the stores of the transmitter acetylcholine are depleted (see Bowman & Marshall, 1972). The action of HC-3 on the transport of choline is most clearly seen on tissue preparations containing intact cells where there are more restrictive barriers to the free movement of choline (Gardiner, 1961).

In the intact animal changes are therefore to be anticipated in the distribution and disposition of free choline during hemicholinium intoxication. Sites or tissues that normally obtain their choline requirements as free choline would be expected to become depleted; those other organs or tissues supplying the base might conversely be expected to show raised levels. Knowledge of the changes should be of assistance in the study of the normal metabolism and function of choline as well as of the details of hemicholinium lethality.

The experiments described here were conducted in rabbits; changes in the plasma and tissue concentrations of endogenous free choline were followed during the development of intoxication with HC-3 or its *p*-terphenyl analogue (TPHC-3) and measurements were made of the disposition of exogenous choline in the presence of HC-3. The tissues studied were plasma and erythrocytes of arterial blood, skeletal muscle, heart, brain, lung, liver, kidney and small intestine. The results of parallel experiments conducted over the same period but without the use of the hemicholinium have been published previously (Gardiner & Gwee, 1974). Preliminary results of this study were presented at the 5th International Congress of Pharmacology (Gardiner & Gwee, 1972).

## Methods

Adult rabbits of either sex weighing 1.3–1.6 kg were used. The animals were anaesthetized with sodium pentobarbitone (50 mg/kg i.v.). The preparation of the animals, general experimental and analytical procedures, and choline bioassay have been described previously (Gardiner & Gwee, 1974).

The hemicholinium HC-3 was prepared as described by Long & Schueler (1954) with modifications introduced by V.B. Haarstadt & F.W. Schueler (personal communication). The *p*-terphenyl analogue of HC-3 (TPHC-3) was prepared similarly (Lee, 1969) and it was used in the form of the monohydrate, recrystallized from ethanol, $C_{30}H_{38}O_4N_2Br_2.H_2O$, molecular weight 668.46.

## Results

### *Plasma choline concentration after hemicholinium treatment*

All six animals given HC-3 0.35 μmol/kg and two out of six given the same dose of TPHC-3 exhibited no signs of intoxication over a 3 h period. The other four rabbits receiving TPHC-3 showed respiratory impairment approximately 60 min after the drug was injected: periods of slow decline of the respiratory amplitude were terminated by a large gasp and followed by a temporary return to normal amplitude. As the intoxication proceeded the gasps became more frequent and the fall in amplitude became quicker; the rate of respiration also decreased. The effect was maximal at 1.5 to 2 h, after which the respiration recovered. The animals were breathing normally at the end of the experiment.

The plasma choline concentration remained steady in the control animals and in those given a hemicholinium whose respiration was unaffected. In the 4 animals which showed respiratory impairment after 0.35 μmol/kg of TPHC-3 there were 2 to 4-fold increases in the plasma choline concentrations during the periods when the respiratory difficulties were most severe. The choline concentrations decreased again as respiration improved.

Table 1 and Figure 1 show the changes in plasma choline concentrations which followed the injection of higher doses of the hemicholiniums sufficient to produce complete or almost complete respiratory failure. In these experiments two intravenous injections of either HC-3 (1.4 μmol/kg) or TPHC-3 (0.7 μmol/kg) were given 1 h apart. In two rabbits, after the first dose, a mild stimulation of the respiration was seen. One hour after the second injection the progressive respiratory distress characteristic of hemicholinium intoxication developed. The respiratory disturbances were followed by a rise in the plasma choline concentration, the terminal values being 8 to 15-fold greater than the basal values. The increase in choline concentration was greatest in those animals whose respiration was most severely affected.

### *Effect of hemicholinium-3 on continuous infusions of choline*

In the experiments described above it appeared that increases in plasma choline concentration which occurred after the administration of a hemicholinium may have been the consequence of the respiratory impairment rather than a direct action of the drug. To investigate whether, in the absence of respiratory distress, HC-3 could cause a rise in plasma choline concentration, choline was infused intravenously in the absence and presence of HC-3. The plasma choline concentrations were measured every 5 minutes. In other experiments, in the absence of a hemicholinium compound, it was observed that the rate of the choline infusion had to be larger than 400 nmol $kg^{-1}$ $min^{-1}$ to cause a significant rise in the plasma choline con-

**Table 1** Plasma choline concentration following intravenous injection of hemicholinium no. 3 (HC-3, 2 × 1.4 μmol/kg) or its *p*-terphenyl analogue (TPHC-3, 2 × 0.7 μmol/kg)

| | *Plasma choline concentration (nmol/ml)* | | | |
|---|---|---|---|---|
| | *Before first injection* | *After hemicholinium* | | |
| | | *1st h* | *2nd h* | *3rd h* |
| | | | *HC-3 (2 × 1.4 μmol/kg)* | |
| 1 | 5.2 ± 0.2 | 6.5 ± 1.1 | 13 ± 5.2 | 40 ± 1.9** |
| 2 | 7.6 ± 1.1 | 10.0 ± 1.0 | 17 ± 1.2** | 49 ± 13** |
| 3 | 8.4 ± 0.9 | 9.5 ± 0.4 | 14 ± 3.1 | 47 ± 5.1** |
| | | | *TPHC-3 (2 × 0.7 μmol/kg)* | |
| 1 | 6.4 ± 0.6 | 8.3 ± 0.3* | 20 ± 2.4** | 63 ± 1.2** |
| 2 | 9.8 ± 0.8 | 13.0 ± 1.6 | 25 ± 6.7 | 69 ± 5.2** |
| 3 | 5.5 ± 0.3 | 6.9 ± 0.5 | 20 ± 6.0 | 77 ± 11** |

After collection of control samples, the first injection of HC-3 or TPHC-3 was given followed by the second injection 1 h later. Each value is the mean ± s.e. mean of four samples taken at 15 min intervals.
Significance of difference from pre-injection value: $^*P < 0.05$; $^{**}P < 0.001$.

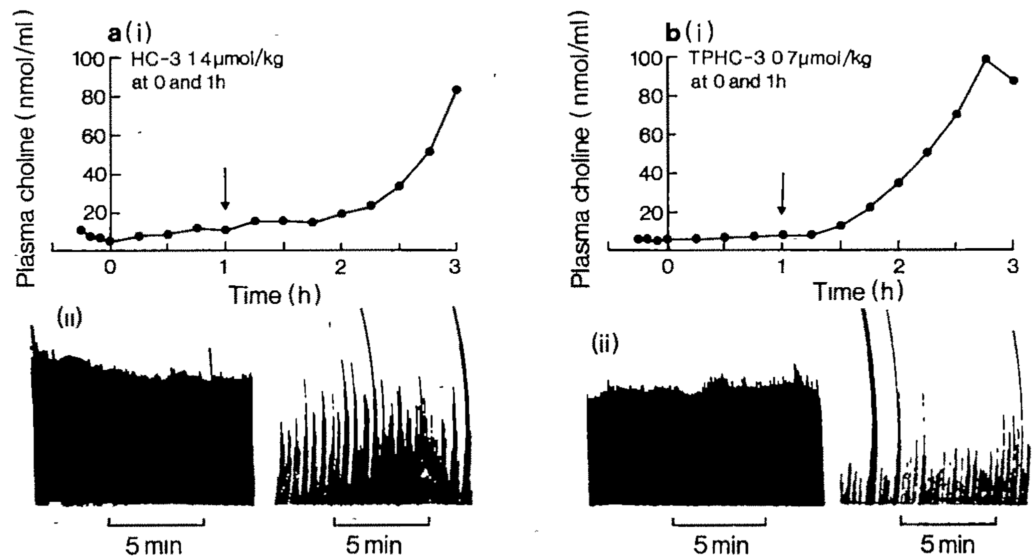

**Figure 1** Plasma choline concentration and respiratory activity of (a) rabbit given hemicholinium no. 3 (HC-3) and (b) another rabbit given its *p*-terphenyl analogue (TPHC-3) (ai) HC-3 (1.4 μmol/kg) injected at zero time and 1 h later at arrow. (bi) TPHC-3 (0.7 μmol/kg) injected at zero time and 1 h later. (ai) and (bi) show the plasma choline concentrations. (aii) and (bii) are examples of the kymograph tracing produced by a piston recorder connected to the side arm of a tracheal cannula. First tracing: respiratory movements during the pre-injection period; second tracing: after the injection when the respiration was most severely affected.

centration (Gardiner & Gwee, 1974). In the present experiments therefore choline was infused at rates of 200, 400, 800 and 1200 nmol kg$^{-1}$ min$^{-1}$ for 15 min each. HC-3 (1.4 μmol/kg) was injected 30 min before the start of the first choline infusion period. The infusions were completed before any depressant effect on the respiration was detectable. After HC-3 (1.4 μmol/kg) had been given, the plasma choline concentration was clearly raised in three rabbits out of four when choline was infused at 200 nmol kg$^{-1}$ min$^{-1}$ and in all four when the rate was 400 nmol kg$^{-1}$ min$^{-1}$ (see Table 2).

**Table 2** Effect of hemicholinium no. 3 (HC-3) on the plasma choline concentration during the intravenous infusion of choline

| | *Plasma choline concentration (nmol/ml)* | | | | |
|---|---|---|---|---|---|
| | *Control* | *$R_1$* | *$R_2$* | *$R_3$* | *$R_4$* |
| | | | *Without HC-3* | | |
| 1 | 21.3 ± 0.9 | 26.0 ± 2.3 | 23.0 ± 1.0 | 36.6 ± 1.0** | 45.2 ± 1.0** |
| 2 | 10.8 ± 1.0 | 11.0 ± 0 | 15.0 ± 0.4** | 23.9 ± 1.7** | 36.5 ± 0.3** |
| 3 | 12.4 ± 0.9 | 14.5 ± 1.7 | 13.9 ± 1.3 | 22.5 ± 0** | 40.0 ± 5.0** |
| 4 | 10.1 ± 0.7 | 11.3 ± 0.3 | 12.7 ± 0.7* | 17.4 ± 0.8** | 31.0 ± 1.0** |
| | | | *With HC-3* | | |
| 5 | 9.4 ± 1.4 | 9.7 ± 0.3 | 23.3 ± 0.3** | 32.9 ± 1.0** | 43.0 ± 1.7** |
| 6 | 6.3 ± 0.2 | 9.2 ± 0.6 | 15.5 ± 1.3** | 21.6 ± 1.3** | 43.7 ± 0.8** |
| 7 | 7.3 ± 0.3 | 8.7 ± 0.3* | 13.5 ± 2.3* | 20.5 ± 0.5** | 34.7 ± 0.3** |
| 8 | 11.9 ± 0.8 | 16.6 ± 1.0* | 26.5 ± 2.2** | 32.2 ± 1.2** | 61.7 ± 1.7** |

Choline was infused for 15 min at each of four rates ($R_1$) 0.2, ($R_2$) 0.4, ($R_3$) 0.8 and ($R_4$) 1.2 μmol kg$^{-1}$ min$^{-1}$. Arterial blood samples were taken at 5 min intervals, four during the control period and three during each of the choline infusions. The values are means ± s.e. mean of all samples taken during each period. In experiments 5 to 8 HC-3 (1.4 μmol/kg) was injected intravenously immediately after the last control sample was taken; the infusion was started 30 min later.

Significance of difference from control values: $^{*}P < 0.05$; $^{**}P < 0.001$.

### *Choline in the erythrocytes*

The concentration of choline in the erythrocytes of arterial blood did not change throughout the experiments in either the control animals or any of those given a hemicholinium.

### *Tissue choline concentrations after hemicholinium treatment*

The concentrations of choline in some tissues of rabbits which were given HC-3 or TPHC-3 (0.35 μmol/kg) and were killed 3 h later are shown in Table 3. Lung choline was raised by about 35% after either HC-3 or TPHC-3 and choline in the kidney was reduced by 30% (TPHC-3) or 40% (HC-3). In the liver only TPHC-3 caused a rise (40%) in the choline concentration. The concentrations of choline in muscle, heart, brain and the terminal portion of the small intestine were unaffected by the administration of either hemicholinium. The choline concentrations in the control rabbits were reported previously (Gardiner & Gwee, 1974). In those experiments it was shown that the delay between the death of the animal and the extraction of tissue samples for choline did not produce any appreciable change in the choline content of the liver, lung and kidney. The results obtained here cannot therefore be accounted for by *post mortem* changes.

## Discussion

In the animals whose respiration was appreciably depressed by the hemicholinium, the 2 to 15-fold rise in plasma choline concentration was unlikely to have been due to gradual accumulation of choline consequent upon a direct inhibitory action of hemicholinium on choline transport mechanisms, as the rise in plasma choline did not precede the development of respiratory difficulty. We consider it likely that hypoxia caused the observed rise in plasma choline concentration. This idea is supported by the experiments of Chan (1971) and Gardiner (1975) in rabbits. They showed that the development of tissue hypoxia produced by the administration of cyanide or of gas mixtures deficient in oxygen, massive haemorrhage, or following respiratory arrest induced by (+)-tubocurarine, was accompanied by rises in the concentration of choline. Gardiner (1975) further showed that the rises following (+)-tubocurarine of TPHC-3 were prevented if the animals were artificially resuscitated.

Our results do not negate the idea that hemicholinium toxicity is primarily due to impaired acetylcholine synthesis at cholinergic junctions. This effect occurring at the neuromuscular junctions of respiratory muscles may well be responsible for, or at least contribute to, the impairment of breathing. However, inhibition of choline transport at such junctions is clearly insufficient to account for the observed rise in plasma choline concentration which, as our experiments show, is secondary to the hypoxia produced.

HC-3 in a dose (1.4 μmol/kg) that did not produce respiratory depression, did not impair the ability of animals to dispose of infused exogenous choline. In HC-3-treated animals the plasma choline concentration started to rise during the infusion of choline 200 nmol kg$^{-1}$ min$^{-1}$ whereas infusion rates of greater than 400 nmol kg$^{-1}$ min$^{-1}$ were required in untreated animals (Gardiner & Gwee, 1974). It is probable therefore that the entry of free choline, from the diet and from sites of synthesis, into the circulation is normally well below a rate of 200 nmol kg$^{-1}$ min$^{-1}$ but that during hemicholinium-induced respiratory distress, the rate of entry of choline from tissues into the circulation exceeds 200 nmol kg$^{-1}$ min$^{-1}$.

The hemicholiniums, at a dose of 0.35 μmol/kg produced variable effects on the tissue disposition of choline. The choline concentration of the brain, intestine, skeletal muscle or heart was not appreciably

**Table 3** Effect of hemicholinium no. 3 (HC-3) or its *p*-terphenyl analogue (TPHC-3) on the concentration of choline in rabbit tissues

| | *Choline concentration (nmol/g tissue)* | | | | | | |
|---|---|---|---|---|---|---|---|
| *Treatment* | *Muscle* | *Heart* | *Brain* | *Lung* | *Liver* | *Ileum*† | *Kidney* |
| Control | 19 ± 2.2 | 84 ± 8 | 200 ± 14 | 320 ± 14 | 390 ± 30 | 430 ± 40 | 500 ± 25 |
| HC-3 0.35 μmol/kg | 12 ± 1.6 | 68 ± 10 | 150 ± 17 | 420 ± 62* | 320 ± 29 | 510 ± 57 | 300 ± 21** |
| TPHC-3 0.35 μmol/kg | 19 ± 2.9 | 100 ± 7 | 210 ± 19 | 410 ± 47* | 560 ± 44** | 520 ± 67 | 360 ± 29** |

In the animals given HC-3 or TPHC-3 (0.35 μmol/kg i.v.) each value is the mean ± s.e. mean from six animals The values for normal animals have been reported previously (Gardiner & Gwee, 1974) and were obtained from analyses on 20 or 26 animals. The animals were killed 3 h after the drug injection. †: terminal small intestine.

Significance of differences from control values: *$P < 0.05$; **$P < 0.001$

affected. This may reflect a lack of susceptibility of the choline uptake mechanisms in these tissues to the action of the hemicholiniums. Alternatively, these tissues may receive their supply of choline not in the free form but, rather, as a derivative whose transport is unaffected by the hemicholinium. There is evidence, for example, that the brain receives choline in a phospholipid form (Björnstad & Bremer, 1966; Ansell & Spanner, 1971). The rise in the choline concentration of the liver caused by TPHC-3 is puzzling.

The kidney choline concentration was significantly decreased by HC-3 (40%) and TPHC-3 (30%). This might be due to the hemicholinium inhibiting the tubular reabsorption of choline in the glomerular filtrate as shown by Vander (1962) and Acara & Rennick (1973). The intracellular choline concentration can then be expected to fall, since the choline will continue to undergo metabolism without being replaced.

Hemicholinium treatment significantly increased the lung choline concentration by about 35%. The lungs, unlike most other tissues, require preformed choline for the synthesis of lecithin (Spitzer, Norman & Morrison, 1969; Gluck, Kulovich, Eidelman, Cordeiro & Khazin, 1972) which forms the major phospholipid component in the lung surfactant system (Klaus, Clements & Havel, 1961; Gluck, Motoyama, Smits & Kulovich, 1967). It is possible that the hemicholinium inhibited the incorporation of choline into lecithin with resultant accumulation of choline in the tissues. An alternative explanation is that the lung normally releases choline into the circulation and that this release process is probably impaired by the hemicholinium, again resulting in accumulation of choline in the tissues. The situation is analogous to the reduced loss of choline from the cerebrospinal fluid in HC-3-treated cats (Gardiner & Domer, 1969). It is not yet possible to distinguish between the two alternatives; although it is known that the lungs readily take up choline from the circulation (Gardiner & Paton, 1972; Gardiner & Gwee, 1974), it is not known how or in what form it is subsequently lost from them.

The effect of the hemicholinium on the disposition of choline in the lung has important implications which might help to explain some of the unusual features seen in hemicholinium intoxication. If the increase in lung choline concentration can be shown to result from inhibition of lecithin synthesis by the hemicholinium, then changes in the surfactant properties of the lung can be expected and this could yet be an additional mechanism contributing to the complex action of the hemicholiniums on the respiration.

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# A POTENT NEW $\beta_2$-ADRENOCEPTOR BLOCKING AGENT

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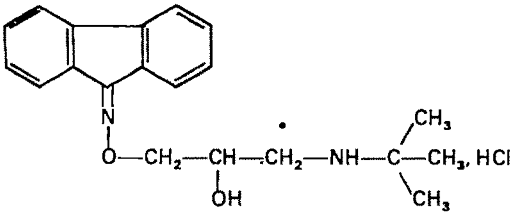
1 (*t*-Butyl-amino-3-ol-2-propyl) oximino-9 fluorene is a new $\beta_2$-adrenoceptor blocking agent with a $pA_2$ of 9.23 ± 0.25 on isolated trachea.

2 It provokes hypertension in normotensive rats and does not prevent arterial hypertension in SHR rats, although it does prevent the renin secretion normally induced by isoprenaline infusion.

## Introduction

The $\beta$-adrenoceptor blocking agents normally used in therapy are characterized by an aminopropanolic side-chain, fixed on phenolic oxygen.

We synthesized a vinylogous series of $\beta$-adrenoceptor blocking agents where the side-chain is carried by an oximic oxygen atom. One of the compounds in this series, IPS 339, hydrochloride of (*t*-butyl-amino-3-ol-2-propyl) oximino-9 fluorene proved interesting.



IPS 339

### *Synthesis of IPS 339*

Fluorenone-oxime was prepared by refluxing overnight 2 equivalents of hydroxylamine hydrochloride with fluorenone in dry pyridine. The sodium salt of the oxime was then reacted in dry dimethylformamide with epichlorhydrin, and the crude epoxide was treated with an excess of tertiobutylamine in ethanol to obtain IPS 339, a yellow powder scarcely soluble in water. It was converted to soluble hydrochloride (m.p. = 160°C) by passing HCl gas through an isopropanolic solution of IPS 339.

Chemical structure and purity were confirmed by elemental analysis; i.r., u.v., n.m.r. analysis and mass spectrometry (Mann, 1975).

## Methods

### *$pA_2$ values*

In vitro *experiments.* Guinea-pigs of either sex weighing between 350 and 550 g, were killed with a sharp blow on the head. Their atria and trachea were quickly excised and excess tissue was removed.

*Isolated atria* (Horii, Kawada, Takeda & Imai, 1974). Atria were divided carefully into right and left halves. The right atrium, which retained spontaneous rhythm, was used to assess the chronotropic action of the drugs. The initial resting tension was set at 0.5 gram. The left atrium, kept at a tension of 0.5 g, was stimulated electrically with square-wave pulses at a frequency of 2.5 Hz with voltages approximately 30% above the threshold (duration 2 milliseconds). The Krebs-Henseleit bathing solution, was kept at 32 ± 1°C, and aerated with a mixture of 95% $O_2$ and 5% $CO_2$. The antagonist was added to the bath 30 min before the assay.

*Isolated trachea* (Levy & Wilkenfeld, 1970). The trachea was cut spirally with a sharp scalpel to produce a thin strip of tracheal tissue. This spiral was cut into two equal segments and both were placed in a bath of Krebs-Henseleit solution. The perfusion fluid contained ascorbic acid (0.1 mg/ml) and phentolamine (0.1 µg/ml). Temperature was maintained at 37°C and the solution was gassed with 95% $O_2$ and 5% $CO_2$. An initial basal tension of 2.5 g was applied to each tracheal strip and the tissue was allowed to stand for 30 min before use. A constant level of tone was maintained by adding carbachol, at a concentration of 0.1 µg/ml to the bath. The carbachol was allowed to act for 15 minutes. Then, without washing, cumulative dose-response curves to isoproterenol were determined before and after treatment with the antagonists. The assay of the antagonist started 60 min after adding it to the bath.

In vivo *experiments.* In all experiments, the antagonists were administered 30 min before isoprenaline.

*Experiments with guinea-pigs.* The animals (400–450 g) were anaesthetized with sodium pentobarbitone (70 mg/kg i.v.).

Resistance to lung inflation (Vargaftig & Coignet, 1969; Konzett & Rössler, 1940) was measured as follows: bronchoconstriction was induced with intravenous 5-hydroxytryptamine (5-HT) administered at 5 min intervals, in doses varying between 1 and 5 µg (but constant for each animal). When a group of three identical responses to 5-HT had been obtained, isoprenaline was administered intravenously and was followed by 5-HT again after 30 seconds. Isoprenaline reduced the bronchial response to 5-HT; doses varying between 0.05 and 0.5 µg were chosen to block 90–95% of the bronchoconstriction. Effective $\beta$-adrenoceptor blocking agents inhibited the antagonism of isoprenaline to 5-HT, and bronchoconstriction, which had previously been blocked, was again present. The percentage of bronchoconstrictor recovery was calculated by comparison of the response to 5-HT before and after administering $\beta$-blocking agents.

Cardiac rhythm was calculated from the blood pressure curve provided by a pressure transducer connected to the carotid artery.

*Experiments with dogs.* Mongrel dogs of either sex, ranging in weight between 8 and 20 kg were premedicated with methotrimeprazine (levomepromazine) (0.5 mg/kg, s.c.) and anaesthetized with sodium pentobarbitone (30 mg/kg, i.v.). The animals were artificially ventilated.

Vascular resistance (Willems & Bogaert, 1975) was measured with a pump placed on a catheter loop and the left femoral artery of the hindleg perfused at constant flow. In order to minimize collateral circulation, the right external iliac artery and the caudal aorta were ligated via a small abdominal incision; the left arteria femoralis profunda was also ligated. Circulation to the paw was occluded by means of a tight ligature. Left femoral, obturator and sciatic nerves were cut. Dogs were heparinized (500 u/kg, i.v.) before starting perfusion. Flow was adjusted to obtain an initial perfusion pressure similar to the systemic pressure. Isoprenaline was injected directly into the catheter. The $\beta$-adrenoceptor blocking agent was injected into the cephalic vein of the right foreleg 30 min before the assay. The perfusion pressure, recorded distally to the pump, was taken as an index of vascular resistance.

Contractile force was measured by means of a strain gauge sutured on the left ventricle.

### *Renin activity*

Five mongrel dogs were anaesthetized with sodium pentobarbitone. Isoprenaline hydrochloride (Isuprel) was infused into the left renal artery at a dose of 0.1 µg $kg^{-1}$ $min^{-1}$ for 10 min before and for 30 min after administration of IPS 339 at the rate of 6.7 µg $kg^{-1}$ $min^{-1}$ for a period of 30 minutes. Renal blood flow was measured with an electromagnetic flow meter. Renin activity was determined by radioimmunoassay on venous renal blood (Imbs, Kraetz, Schmidt, Desaulles & Schwartz, 1975). Blood pressure and left ventricular $dP/dt$ were measured with a transducer. The blockade of $\beta_2$-adrenoceptors was tested by injection of isoprenaline into the renal artery at a dose of 0.25 µg/kg.

### *Blood pressure variations in rats*

Systolic blood pressure was measured with pressure cuffs on the tails of conscious rats.

1. Blood pressure was determined in normotensive rats, 1, 2 and 3 h after oral intake of IPS 339 with three different doses. For each dose, 10 rats were used.
2. Blood pressure was measured in 18 male and 15 female, 6 week-old Okamoto spontaneously hypertensive rats (SHR) divided at random into 3 groups. Intake of IPS 339 (dissolved in 0.9% w/v NaCl solution, 5 ml/kg) took place 6 days out of 7. Systolic blood pressure was measured 24 h after drug intake. The 10 µmol/kg dose of IPS 339 inhibited 23% of the chronotropic effect of intraperitoneal isoprenaline (0.01 µmol/kg) while the 170 µmol/kg dose inhibited 79%.

### *Local anaesthetic and antiarrythmic activity*

*Surface anaesthesia technique.* To determine local anaesthetic activity we used the technique of Bartsch & Knopf (1970) for measurement of surface anaesthesia of the rabbit cornea.

*Antiarrythmic activity.* For *in vitro* studies we used a modification of the technique of Dawes (1946). The guinea-pig atria was electrically stimulated at increasing frequencies and we measured the rhythm at which escape appeared.

The aconitine-induced arrythmias technique of Vargaftig & Coignet (1969) was used for *in vivo* studies.

## Results

IPS 339 *in vitro* was 155 times more active on the trachea than on the atria of the guinea-pig; $pA_2$ for

**Table 1** $\beta$-adrenoceptor blocking effect of IPS 339, practolol and butoxamine *in vitro* and *in vivo*

| | | *Isolated atria* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *$\beta$-blocking agent* | | *Inotropic effect* | *Chronotropic effect* | *Isolated trachea* | *Resistance to lung inflation* | *Cardiac rhythm* | *Vascular resistance* | *Contractile force* |
| IPS 339 | $pA_2 \pm$ s.d. | 7.03 ± 0.16 | 7.04 ± 0.24 | 9.23 ± 0.25 | 7.45 ± 0.14 | 6.03 ± 0.08 | 7.48 ± 0 21 | 6.12 ± 0.17 |
| | | (15) | (10) | (25) | (6) | (7) | (7) | (7) |
| | Slope ± s.d. | −1.49 ± 0.19 | −1.32 ± 0.25 | −0.66 ± 0.09 | −1.28 ± 0 35 | −1.39 ± 0.18 | −1.23 ± 0.25 | −1.46 ± 0.38 |
| Practolol | $pA_2 \pm$ s.d. | 6.77 ± 0.31 | 6.85 ± 0.12 | 5.13 ± 0.30 | 4.60 ± 0.07 | 6.54 ± 0 14 | 4.51 ± 0.11 | 6.45 ± 0.10 |
| | | (8) | (10) | (12) | (5) | (7) | (5) | (5) |
| | Slope ± s.d. | −1.12 ± 0.26 | −1.03 ± 0.09 | −0.51 ± 0.12 | −0.43 ± 0.11 | −1.14 ± 0.18 | −0.95 ± 0.25 | −1.07 ± 0.08 |
| Butoxamine | $pA_2 \pm$ s.d. | 4.82 ± 0.08 | 5.34 ± 0.07 | 6.44 ± 0.37 | 5.04 ± 0.06 | 5.25 ± 0.08 | 5.16 ± 0.09 | — |
| | | (12) | (10) | (8) | (7) | (5) | (6) | |
| | Slope ± s.d | −1.64 ± 0.26 | −1.17 ± 0.10 | −0.83 ± 0.17 | −0.74 ± 0.13 | −0.85 ± 0.12 | −1.50 ± 0.30 | — |

Figures in parentheses show number of measurements taken.

inotropic and chronotropic effects were similar. *In vivo*, its $\beta_2$-adrenoceptor selectivity was 23 in dog experiments and 26 in guinea-pig experiments. The $\beta$-selectivity is expressed as the antilog of the differences between the $pA_2$. Table 1 shows the various $pA_2$ studied and also, for reference, our results with practolol (a $\beta_1$-adrenoceptor blocking agent) and butoxamine, the first $\beta_2$-blocking agent to be described. We have observed that for each one, selectivity is not the same *in vitro* as *in vivo*. Practolol's $\beta_1$-adrenoceptor selectivity was 52 *in vitro*, and 89 *in vivo* for the dog. Butoxamine's $\beta_2$-adrenoceptor selectivity was 13 *in vitro* and only 2 *in vivo*. Slopes of the regression lines, were, for the most part, not significantly different from one. It should be pointed out that our experiments were limited in number and also were carried out in the absence of drugs which could prevent aspecific uptake.

IPS 339's local anaesthetic effect was 36 times less than that of (±)-propranolol. The antiarrythmic effect of IPS 339 *in vitro* was the same as that of propranolol. IPS 339 was more potent, mole per mole *in vivo*, in preventing aconitine ventricular fibrillation than quinidine or lidocaïne. It was also significantly more active than propranolol.

Given orally (Table 2) to six-week-old spontaneously hypertensive rats (SHR) each day for six weeks, IPS 339, in $\beta_2$-adrenoceptor blocking doses (10 μmol/kg) and in doses 17 times higher (170 μmol/kg) did not prevent the occurrence of arterial hypertension, whereas, under these conditions, the $\beta_1$ selective and the non-specific $\beta$-adrenoceptor antagonists do prevent it (Weiss, Lundgren & Folkow, 1974). Given orally to conscious SHR at the same two doses, IPS 339 induced an increase in blood pressure which lasted for 6 hours. The increase did not seem dose-dependent (Table 3).

Given orally to conscious normotensive rats, IPS 339 produced a dose-dependent increase in blood pressure which was highest after 2 h (Table 4).

IPS 339 partially inhibited the isoprenaline-induced increases, both in renal blood flow (RBF) and in renal venous renin activity (PRA) (Table 5). Nevertheless, IPS 339 did not decrease either the heart rate, or $dP/dt$ (the difference for this parameter is not significant), or systemic blood pressure.

## Discussion

IPS 339 is a new potent $\beta_2$-adrenoceptor blocking agent. Butoxamine was the first $\beta_2$-blocker described. Later H 35/25 was believed to have a predominant action on the peripheral adrenoceptors. According to Bristow, Sherrod & Green (1970) its selectivity may be related to factors other than differences in receptors. Recently, Todd (1976) suggested that

**Table 2** Influence of IPS 339 on the occurrence of arterial hypertension in young spontaneously hypertensive rats (SHR)

| | | *Systolic blood pressure (mmHg $\pm ts/\sqrt{n}$)* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *Weeks of experiment* | | | | | |
| *IPS 339 (μmol/kg)* | n *(number of animals)* | *Control* | 1 | 2 | 3 | 4 | 5 | 6 |
| Solvent | 11 | 116.4 ± 11.2 | 123.7 ± 6.7 | 135.9 ± 7,3* | 139.1 ± 4.7** | 151.3 ± 10.1** | 151 8 ± 10.7** | 155.9 ± 13.9** |
| 10 | 11 | 114.1 ± 7.3 | 120.0 ± 5.2 | 129.6 ± 8.7* | 137.7 ± 6.2*** | 145.5 ± 10.6*** | 145.6 ± 7.8*** | 157.7 ± 11.7*** |
| 170 | 11 | 111.4 ± 11.7 | 121.7 ± 10.5<br>($n = 9$) | 135.0 ± 7.4*<br>($n = 8$) | 140.8 ± 6.1*<br>($n = 6$) | 149.0 ± 11.1**<br>($n = 5$) | 153.8 ± 15.0*<br>($n = 4$) | 163.3 ± 51.7<br>($n = 3$) |

Averages statistically different from the control value; matching *t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

**Table 3** Increase in systolic blood pressure of conscious spontaneously hypertensive rats (SHR) after IPS 339 intake

| | | *Systolic blood pressure (mean $\pm ts/\sqrt{n}$)* | | | | | |
|---|---|---|---|---|---|---|---|
| *IPS 339 μmol/kg* | *Control (mmHg)* | *% increase in relation to control (hours after intake)* | | | | | |
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 10 | 132.0 ± 5.6<br>(10) | 13.7 ± 10.5<br>(9) | 16.0 ± 16*<br>(6) | 18.0 ± 12.3**<br>(6) | 13.2 ± 5.6***<br>(9) | 13 4 ± 8 4**<br>(9) | 6.4 ± 5.7**<br>(6) |
| 170 | 143.0 ± 2.9<br>(10) | 14.1 ± 7.8**<br>(9) | 21.4 ± 5.6***<br>(7) | 14.3 ± 9.1*<br>(6) | 11.5 ± 5.4*<br>(8) | 9.6 ± 6 1*<br>(7) | 5.8 ± 5.3*<br>(8) |

Figures in parentheses show number of measurements taken.

Averages statistically different from those with the solvent; matching *t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}001$.

erythro- and threo-alpha methyl propranolol were more active as vascular rather than as cardiac antagonists. Their selectivity seems to be approximately 10 *in vivo*. They are approximately as active on $\beta_2$-receptors as propranolol. IPS 339 is more selective and more potent than propranolol.

For IPS 339, and also for butoxamine and practolol, we obtained not only their *in vitro* $pA_2$ but also their apparent *in vivo* $pA_2$. We do not think it is strictly permissible to use *in vivo* injected doses to obtain Schild plots for $pA_2$ determination. However, in our experiments (Belhadj-Mostefa, 1975) and from Table 1, we can observe the strict similarity of the 'apparent *in vitro* $pA_2$' of IPS 339, not only on two species but also on two different tissues. On the other hand we have no explanation for the difference of *in vitro* and *in vivo* selectivities. Biodisposability could account for this difference, but this is not borne out by our *in vivo* results.

IPS 339 raises blood pressure in conscious, normotensive or SH rats and does not prevent the occurrence of hypertension in young SHR. $\beta$-Blockers generally have an opposite effect. Regoli, Regoli & Gysling (1971) have shown that in urethane anaesthetized rats, intravenous injections of oxprenolol, or propranolol increase arterial pressure, while practolol is ineffective. This pressor effect is not entirely due to the elimination of the $\beta$-stimulation of exogenous adrenaline on the vascular system.

Our experiments give no clues as to the explanation of the hypertension observed. From experiments on anaesthetized dogs, it seems that even at $\beta_2$-blocking doses, IPS 339 depresses the renin secretion normally induced by isoprenaline. This point needs to be verified extensively. Firstly the nature of $\beta$-receptors involved in the control of renin secretion are still subject to discussion. Secondly, our results suggest that the decrease in renin activity cannot account for the fall in blood pressure observed with non-selective beta-blockers.

**Table 4** Increase in systolic blood pressure of normal conscious rats after IPS 339 intake

| | | *Systolic blood pressure (mean ± ts/$\sqrt{n}$)* | | |
|---|---|---|---|---|
| *IPS 339 (µmol/kg* | *Control (mmHg)* | *% increase in relation to control (hours after intake)* | | |
| | | 1 | 2 | 3 |
| 10 | 118.0 ± 4.2 | 9.1 ± 3.6 | 10.0 ± 5.1 | 3.6 ± 3.5 |
| 30 | 98.5 ± 5.0 | 12.0 ± 7.3 | 14.4 ± 5.2 | 12.9 ± 7.6 |
| 100 | 92.0 ± 7.6 | 16.9 ± 8.7 | 21.9 ± 8.1 | 19.6 ± 9.3 |

For each dose 10 rats were used.
Variation of pressure are dose-dependent at 2 and 3 h (regression analysis).

**Table 5** Effects of isoprenaline before and after treatment with IPS 339 (5 anaesthetized dogs)

| | *Control values* | *After IPS 339* |
|---|---|---|
| Heart rate (per min) | 134 ± 12.5 | 139 ± 12.5 |
| d*P*/d*t* max (mmHg/s) | 2333 ± 391 | 2016 ± 194 |
| Mean BP (mmHg) | 125 ± 8.6 | 120 ± 7.2 |
| % rise in RBF during isoprenaline infusion | 14.7 ± 2.3 | 4.9 ± 0.9 |
| % rise in renal venous PRA | 111 ± 31 | 26 ± 20 |

PRA = plasma renin activity; RBF = renal blood flow.

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# THE EFFECTS OF MORPHINE ON THE ACCUMULATION OF HOMOVANILLIC AND 5-HYDROXYINDOLEACETIC ACIDS IN THE CHOROID PLEXUS OF RATS

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1 Choroid plexus obtained from the lateral ventricles of the rat actively accumulated homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA).

2 Morphine $5\times10^{-6}$M to $5\times10^{-4}$M potentiated 5-HIAA accumulation but did not affect HVA accumulation. Levorphanol and dextrorphan had little effect.

3 Naloxone at high concentrations inhibited both HVA and 5-HIAA accumulation.

4 Glutamic acid, glycine, and arginine also decreased 5-HIAA accumulation, but lysine, tryptophan, and aspartic acid had no effect.

5 Probenecid, naloxone, arginine, glycine, and tryptophan blocked the increase of 5-HIAA accumulation induced by morphine.

6 Acute or chronic morphine treatment did not increase the accumulation of 5-HIAA.

7 These results suggest that the increase of 5-HIAA or HVA in brain by morphine is not due to the inhibition of the elimination of these metabolites from the choroid plexus.

## Introduction

Several neurotransmitters have been implicated in the pharmacological actions of morphine, and numerous attempts have been made to elucidate the mechanism of action of morphine involving changes in levels of biogenic amines such as 5-hydroxytryptamine (5-HT) or catecholamines (see reviews of Way & Shen, 1971; Takemori, 1974, and many others). Since the turnover of biogenic amines in neurones may reflect neuronal activity, the effects of morphine on their turnover have been studied by a variety of methods.

However, the results of these investigations are often equivocal because different methods were used. One method of studying the turnover of 5-HT is to measure 5-hydroxyindoleacetic acid or homovanillic acid accumulation after inhibiting their transport by probenecid (Neff, Tozer & Brodie, 1967). This method has its advantages; it is simple and can be applied to human beings. However, if a drug *per se* has an effect on the transport of acidic metabolites in the brain, the use of this method to study 5-HT or dopamine turnover may produce misleading results. Since the choroid plexus is considered to be one of the active sites of their transport, and accumulation of acidic metabolites in the choroid plexus *in vitro* could reflect such activity, we examined the effects of morphine in this system. This choice was supported by the fact that, after the injection of morphine, histological examination of the choroid plexus revealed the appearance of numerous vacuoles, suggesting intense secretory activity and pronounced ischaemia (Wajda, Wajda, Manigault & Steiner, 1974).

## Methods

5-Hydroxyindoleacetic acid (5-HIAA), labelled with $^{14}$C in the carboxyl position (specific activity 22.1 mCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass. [G-$^3$H]-homovanillic acid (specific activity 585 mCi/mmol) was obtained from Commissariat a l'Energie Atomique, Gif-sur-Yvette, France. The organic solvents which dissolved these radioactive compounds were removed by vacuum evaporation, and subsequently the compounds were dissolved in 0.01 N HCl or distilled water. The incubation medium was modified Krebs-Ringer phosphate solution of the following composition (mM): NaCl 135, KCl 5, $MgSO_4$ 1.3, $CaCl_2$ 0.5, glucose 12, and $NaH_2PO_4$-$Na_2HPO_4$ buffer 10. This incubation medium has been used in previous studies dealing with accumulation in the choroid plexus of morphine (Takemori & Stenwick, 1966) and of methadone (Huang & Takemori, 1975). The incubation medium was bubbled with $O_2$ for 1 h before use; the pH of the medium was 7.4. 5-HIAA or homovanillic acid (HVA) and other chemicals were added to 3 ml of incubation medium just before incubation.

Female Sprague-Dawley rats (200 to 250 g) were used in all experiments. The schedule of chronic morphine treatment was adapted from Cox, Ginsburg, Willis & Davies (1975). Rats were treated with morphine sulphate twice daily for up to 7 days. Rats were decapitated 1 to 6 h after the last dose of morphine (200 mg/kg), and their brains rapidly exposed. Before incubation, the choroid plexus was isolated and placed in a cold moist glass chamber with filter paper soaked with 0.9% w/v NaCl solution (saline). Not more than 5 min elapsed from the isolation of the choroid plexus to the incubation. The incubation was performed at 37°C; the water bath was shaken at 60 rev/minute. Air was replaced by $O_2$, and after various time intervals the choroid plexus was removed from the medium and washed twice with ice-cold non-radioactive medium; the excess of medium in the choroid plexus was removed by drawing it across a glass slide as described by Cserr & Van Dyke, 1971. After weighing, the choroid plexus was transferred to the counting vials and the tissue dissolved in 0.5 ml of Soluene (Packard Inst. Co., Downer Grove, Illinois, U.S.A.). Their radioactivity and the radioactivity of the incubation media were measured by liquid scintillation spectrometry. Scintillation fluid consisted of PPO (5 g) and dimethyl POPOP (0.25 g) in 1 l of toluene. The counting efficiency was about 85% for $^{14}C$ and 30% for $^{3}H$.

The calculation of 5-HIAA accumulation (tissue to medium ratio) was d/min (disintegrations per minute) per wet choroid plexus weight (g) divided by d/min of incubation medium (ml) and multiplied by the factor of 0.8 (Quay, 1966). Neither metabolism nor the binding of 5-HIAA in the choroid plexus was considered (Sampath & Neff, 1974). HVA was not metabolized significantly by the choroid plexus during 60 min incubation. The data were statistically analyzed by Student's *t* test (paired and two tail) because control and experimental choroid plexus were from the same rats. Significant value is *P* less than 0.05.

## Results

### *Accumulation of 5-hydroxyindoleacetic and homovanillic acids by the choroid plexus*

Choroid plexus from rat lateral ventricle accumulates 5-HIAA and HVA against an apparent concentration gradient, as indicated by the fact that tissue to medium ratio was always greater than unity during the incubation period (5–60 minutes). The accumulation of 5-HIAA increased continuously with the incubation time while HVA accumulation was maximal at about 30 min then gradually decreased so that tissue to medium ratio after 60 min was similar to that at 5 minutes.

In the subsequent experiments the incubation times for 5-HIAA and HVA were 20 and 15 min

**Table 1** Effect of opiates on the accumulation of 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) in choroid plexus of rat

| *Compound* | *Final concentration* (M) | *Mean % of control* 5-HIAA | *Mean % of control* HVA |
|---|---|---|---|
| Morphine | $5 \times 10^{-3}$ | — | 105.7 ± 6.5 |
| | $5 \times 10^{-4}$ | 122.2 ± 6.8* | 101.1 ± 8.0 |
| | $5 \times 10^{-5}$ | 136.1 ± 5.5* | 100.8 ± 6.3 |
| | $5 \times 10^{-6}$ | 119.4 ± 4.2* | — |
| Levorphanol | $5 \times 10^{-4}$ | 100.3 ± 6.0 | 86.6 ± 6.8 |
| | $5 \times 10^{-5}$ | 113.3 ± 2.0* | 88.6 ± 12.4 |
| | $5 \times 10^{-6}$ | 104.8 ± 6.6 | — |
| Dextrorphan | $5 \times 10^{-4}$ | 97.6 ± 5.1 | 96.3 ± 7.0 |
| | $5 \times 10^{-5}$ | 107.3 ± 4.6 | 93.1 ± 5.9 |
| Naloxone | $5 \times 10^{-3}$ | — | 62.6 ± 7.2* |
| | $5 \times 10^{-4}$ | 83.4 ± 4.6* | 82.5 ± 4.5* |
| | $5 \times 10^{-5}$ | 89.4 ± 8.6 | 101.7 ± 10.4 |
| | $5 \times 10^{-6}$ | 97.4 ± 3.1 | — |

The accumulation was measured by tissue to medium ratio of choroid plexus incubated in $3.6 \times 10^{-7}$ M of 5-HIAA for 20 min and $2.7 \times 10^{-7}$ M of homovanillic acid for 15 min at 37°C under $O_2$. Each value represents a mean with s.e. mean of five paired experiments.

* $P < 0.05$.

respectively since they coincided with the middle of the linear increase of accumulation.

### *Effect of opiates on the accumulation of 5-hydroxyindoleacetic and homovanillic acids* in vitro

In the presence of morphine in the incubation medium ($5 \times 10^{-6}$ to $5 \times 10^{-4}$ M) the accumulation of 5-HIAA in the choroid plexus increased significantly (about 30%). Levorphanol was less effective and dextrorphan was without any effect. In the presence of naloxone ($5 \times 10^{-4}$ M) the accumulation of 5-HIAA was decreased. The effect of opiates on the uptake of HVA by choroid plexus was different from the effect of 5-HIAA uptake. Morphine, levorphanol, and dextrorphan were without any effect. Naloxone at high concentrations inhibited the accumulation of HVA (Table 1).

### *Pretreatment with morphine and the accumulation of 5-hydroxyindoleacetic acid*

Choroid plexus obtained from rats injected with a single dose of morphine (10 or 40 mg/kg) and killed 1 h after the injection showed no difference from that of saline-treated rats; in the two groups the tissue to medium ratio after 3 or 20 min incubation was the same. After morphine injections, the incubation with probenecid resulted in a decreased uptake (Table 2). After chronic morphine treatment for one week and

**Table 2** Accumulation of 5-hydroxyindoleacetic acid (5-HIAA) in the choroid plexus isolated from morphine-treated rats

| *Experimental conditions* | *Incubation time (min)* | *T/M ± s.e.** |
|---|---|---|
| Control (saline-treated) | 3 | 1.6±0.1 |
| | 20 | 3.7±0.2 |
| Morphine 10 mg/kg for 1 h | 3 | 1.6±0.1 |
| | 20 | 3.8±0.2 |
| Morphine 40 mg/kg for 1 h | 20 | 3.9±0.1 |
| Morphine 40 mg/kg for 1 h and incubation with $1 \times 10^{-4}$ M of probenecid | 20 | 2.6±0.1** |
| Morphine 20 mg/kg increased to 200 mg/kg twice daily for 7 days; 1 h after last dose | 20 | 3.9±0.4 |
| Morphine 20 mg/kg increased to 200 mg/kg twice daily for 7 days; 6 h after last dose | 20 | 4.0±0.2 |

*Tissue to medium (T/M) ratio was based on the uptake of $3.6 \times 10^{-7}$ M of 5-HIAA for 20 min at 37°C under $O_2$. Each value represents a mean with s.e. mean of four experiments.

**$P < 0.05$.

**Table 3** Effect of amino acids and probenecid on the accumulation of 5-hydroxyindoleacetic acid (5-HIAA) in the choroid plexus

| *Amino acid* | *Tissue to medium ratios* | | |
|---|---|---|---|
| | *Control* | *With amino acid** | *Mean % of control* |
| Arginine | 3.8±0.2 | 2.9±0.1 | 76.9** |
| Lysine | 3.8±0.4 | 3.6±0.1 | 94.7 |
| Glycine | 4.0±0.3 | 3.1±0.2 | 77.5** |
| Tryptophan | 3.9±0.1 | 4.0±0.3 | 102.2 |
| Aspartic acid | 3.6±0.3 | 3.3±0.1 | 90.7 |
| Glutamic acid | 4.0±0.4 | 3 4±0.2 | 85.0** |
| Probenecid | 3.9±0.1 | 2.6±0.1 | 66.7** |

*The final concentration of amino acids was $1 \times 10^{-4}$ M. Each value represents mean with s.e. mean of five experiments.

**$P < 0.05$.

also upon withdrawal from morphine, the choroid plexus showed the same accumulation ratio as the controls.

### *Effects of amino acids*

Six amino acids were tested for their effects on the accumulation of 5-HIAA by the choroid plexus. Arginine, glycine, and glutamic acid were inhibitory at a concentration of $1 \times 10^{-4}$M (about 20%), whereas lysine, tryptophan, and aspartic acid were without effect (Table 3). The results suggest that the inhibitory effect of amino acids on the accumulation of 5-HIAA does not depend on the pH of the amino acid. The effect of amino acids was not present at low concentrations; for instance, glycine at a concentration of $1 \times 10^{-6}$M was without effect.

### *Interaction of morphine and other compounds on 5-hydroxyindoleacetic acid accumulation*

Naloxone, aspartic acid, and tryptophan were tested together with morphine at concentrations that were without effect in the absence of morphine. However, they were able to block the increase in the accumulation of 5-HIAA induced by morphine alone (Table 4). Probenecid and glycine which decreased the accumulation of 5-HIAA (Table 3) inhibited the usual increase in accumulation induced by morphine.

## Discussion

The present results indicate that the choroid plexus isolated from the rat lateral ventricles can actively accumulate 5-HIAA and HVA and these uptake mechanisms differ in their response to morphine which potentiated the accumulation of 5-HIAA but had no effect on the accumulation of HVA. However, the reasons for the differences and for the appearance of the vacuoles in the ependymal cells of the choroid plexus after morphine were not elucidated by this investigation.

Since levorphan, a more potent analgesic than morphine, was less potent in stimulating the accumulation of 5-HIAA, it seems that the potentiation of 5-HIAA accumulation has no relation to the analgesic effects of opiates. Nevertheless, naloxone, which antagonizes the analgesic action of opiates, was able to block this potentiation of 5-HIAA accumulation produced by morphine.

Goodlet & Sugrue (1974) reported that morphine was able to produce an increase in the concentration of 5-HIAA in the brain, whereas other narcotics such as methadone, pentazocine, and pethidine or naloxone were inactive. They suggested that only morphine is involved in serotoninergic activity of the brain. More recent study of inhibitory effect of fluoxetine on 5-HT uptake further supports their conclusion (Sugrue & McIndewar, 1976). Our results also support this conclusion because among four morphine analogues studied, only morphine increased the accumulation of 5-HIAA in the choroid plexus.

We have confirmed that probenecid was very effective in inhibiting 5-HIAA uptake in the isolated choroid plexus; it also inhibited the accumulation of 5-HIAA in the choroid plexus obtained from morphine-treated rats. This result would be expected if the increase of 5-HIAA by morphine were due to the inhibition of its transport since acute and chronic morphine treatments potentiate the increase of 5-HIAA in rat brain by probenecid (Haubrich & Blake, 1973; Papeshi, Theiss & Herz, 1974; Goodlet & Sugrue, 1974).

The increase of 5-HIAA accumulation in isolated choroid plexus by morphine seems to be incompatible with the increase of 5-HIAA concentration in brain

**Table 4** The interaction of morphine with other compounds in the accumulation of 5-hydroxyindoleacetic acid in the choroid plexus

| *Treatment with compound** | *Tissue to medium ratios* | | |
|---|---|---|---|
| | *Morphine alone* | *Morphine + compound* | *Mean % of morphine* |
| Probenecid | 4.6 ± 0.2 | 2.6 ± 0.3 | 56.8** |
| Naloxone | 4.5 ± 0.1 | 3.8 ± 0.2 | 86.1** |
| Aspartic acid | 4.3 ± 0.4 | 3.5 ± 0.4 | 79.8** |
| Glycine | 4.2 ± 0.2 | 3 7 ± 0.2 | 87.9** |
| Tryptophan | 4.7 ± 0.4 | 3.6 ± 0.2 | 75.9** |

Morphine was present in all incubations.

*The concentration of morphine and naloxone was $5 \times 10^{-6}$M; other compounds were $1 \times 10^{-4}$M. The conditions of incubation were as in Table 1. Each value represents mean with s.e. mean of five experiments

**$P < 0.05$.

after acute or chronic morphine injection. Since the choroid plexus is one of the active sites for the elimination of 5-HIAA from the brain, morphine would be expected to act like probenecid and inhibit 5-HIAA accumulation in choroid plexus. It is possible that morphine inhibits other sites of the probenecid-sensitive transport system; it is known that the elimination of 5-HIAA by choroid plexus represents a minor process (Meek & Neff, 1973). The location of this probenecid-sensitive site is not likely to be in neurones, because Forn (1972) demonstrated that probenecid could not inhibit the efflux of 5-HIAA in brain slices. It is possible that the active site is located in brain capillaries.

Morphine-induced increase in 5-HIAA levels in the brain seems to be due to an increased rate of 5-HIAA formation rather than an inhibition of 5-HIAA efflux from brain (Haubrich & Blake, 1973; Goodlet & Sugrue, 1974). Our finding of an increase in the accumulation of 5-HIAA in the choroid plexus produced by morphine is in agreement with results of previous authors.

Since amino acids are present in the cerebrospinal fluid and certain amino acids, e.g. tryptophan (Ho, Brase, Loh & Way, 1975), aspartic acid (Koyuncuoglu, Gungor, Sagduyu & Eroglu, 1974), and glycine (Stern & Stern, 1974) prevent and modify morphine actions and physical dependence, the effects of these amino acids on 5-HIAA accumulation in choroid plexus were studied. At high concentrations, not far from the physiological concentration in cerebrospinal fluid, these amino acids decreased the accumulation of 5-HIAA and also blocked the increase of 5-HIAA accumulation produced by morphine. These results suggest that morphine cannot increase 5-HIAA uptake into the choroid plexus because of the presence of amino acids in the cerebrospinal fluid.

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# ANAPHYLACTIC REACTIONS TO ENDOTOXIN IN GUINEA-PIG TISSUES: RELATIONSHIP TO ENDOTOXIN TOXICITY

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1 A lipopolysaccharide extract of *Escherichia coli* 026:B6 cells (026:B6(B) endotoxin) was shown to be toxic to normal adult guinea-pigs.

2 The agent had no action on isolated preparations of ileum and heart taken from normal adult guinea-pigs.

3 Ileal segments from animals actively immunized against 026:B6(B) endotoxin showed dose-dependent contractions when exposed to endotoxin. Desensitization phenomena were demonstrated.

4 Reactivity of 026:B6(B) endotoxin was transferred to isolated preparations of ileum and heart from normal animals by passive transfer of immune serum.

5 Tissue responses to 026:B6(B) were associated with release of ileal spasmogen into the bath medium. Mepyramine blocked the effects of this spasmogen at bath concentrations which caused little change in ileal responses to carbachol.

6 It is concluded that *E. coli* endotoxin can elicit anaphylactic reactions, and that this process may potentiate endotoxin toxicity in sensitized animals. However, endotoxin toxicity in guinea-pigs does not appear to depend on this kind of allergic process.

## Introduction

Lipopolysaccharide complexes, termed endotoxins, may be derived from the cell walls of gram-negative bacteria by boiling or by chemical methods. (Luderitz, Westphal, Staub & Nikaido, 1971). These extracts produce a diverse range of biological effects when injected into mammals, including lethal cardiovascular disorganization (shock) (Bennett & Cluff, 1957; Gilbert, 1960; Raskova & Vaneček, 1964; Hinshaw, 1971; Milner, Rudbach & Ribi, 1971). It is thought that these agents produce the shock state associated with sepsis in man.

The mechanisms of action of these endotoxins on the circulation remain unknown. Suggested mechanisms range from direct pharmacological effects on smooth muscle (Urbaschek, 1964; Zweifach, 1964; King & Cox, 1972) and interactions with autacoid mechanisms (Zweifach, 1964; Urbaschek & Versteyl, 1965; Hinshaw, 1971) to immunologically mediated allergic reactions such as anaphylaxis (Chedid & Parant, 1971).

Recent experiments with guinea-pigs have provided evidence that a range of endotoxins produce lethal effects, yet are inert on standard pharmacological preparations utilizing guinea-pig tissues including gut, ureter, vas deferens, portal vein, uterus and atria (McLean, unpublished results). These studies also established that there was no interaction with $\alpha$-adrenoceptor or $H_1$ and $H_2$-histamine receptor mechanisms. Consequently it was of interest to test the possibility that anaphylactic mechanisms were involved in this species, as this could be carried out free from interactions with the other proposed mechanisms described above.

Since the classical work of Schultz (1910) and Dale (1912–1913), it has been recognized that the anaphylactic process may be studied under *in vitro* conditions (tissue anaphylaxis). Using these methods many properties of the anaphylactic process have been defined (Mongar & Schild, 1962). However, the basic properties are those described by Dale as follows. Exposure of sensitized tissues to a specific antigen results in release of pharmacologically active agents; repeat exposure elicits a smaller reaction than the initial challenge (desensitization); reactivity to the antigen may be transferred to normal tissues through transfer of serum from an immunized animal.

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In this study, the effect of a trichloracetic acid extract of *Escherichia coli* 026:B6 cells (026:B6(B) endotoxin) on isolated tissues from normal and immunized guinea-pigs was investigated. A parallel study was made of toxicity of this extract in normal adult guinea-pigs.

## Methods

### *Endotoxic entract*

A trichloracetic acid extract of *E. coli* 026:B6 cells was purchased from Difco Laboratories. Test solutions of this endotoxin were freshly prepared from stock solutions in distilled water. This extract is termed 026:B6(B) in the text.

### *Toxicity testing*

Normal adult guinea-pigs were injected intravenously (i.v.) and into the peritoneum (i.p.) with 026:B6(B) at a dose of either 0.5 mg/kg or 2 mg/kg.

### *Active immunization schedules*

Animals were actively immunized according to one of two schedules, termed A and B in the text.

*Schedule A.* A single intraperitoneal injection of 2 mg/kg of 026:B6(B) was given. Survivors were studied four weeks after inoculation. A pool of serum was prepared after bleeding five animals using conventional techniques.

*Schedule B.* Footpads were injected with 0.4 ml of a 1:1 mixture of complete Freund's Adjuvant and a 2.5 mg/ml solution of 026:B6(B) in distilled water. From the fourteenth day after 'priming', the animals were 'boosted' every second day by intradermal injections into the mantle region, of 20 µg 026:B6(B) in 0.9% w/v NaCl solution (saline). Four injections were given before the animals were killed. A pool of serum was made from five immunized animals.

### *Passive immunization*

Normal animals were given an intraperitoneal injection of 4 ml/kg of immune serum 16 h before ileal segments were removed and exposed to endotoxin (see below).

### *Isolated ileal preparations*

Ilea were removed from normal and immunized animals after stunning and bleeding. Segments were mounted in an isolated organ bath apparatus and bathed with modified Tyrode solution (Feigen, Vaughan Williams, Peterson & Nielsen, 1960) and attached to an isotonic lever writing on a smoked drum apparatus. Dose-response characteristics to histamine were determined over the range $10^{-9}$ to $10^{-7}$ M. Preparations were exposed to 026:B6(B) by cumulative addition to the bath over a test range defined by screening studies on one ileal segment from each animal. Screening doses ranged from 0.1 to 500 µg/ml. When a reaction range was defined, responses were quantitated in terms of histamine equivalents (Feigen *et al.*, 1960) in two additional segments from each animal. The mean was calculated. All ileal experiments were carried out with 2 µg/ml of semicarbazide in the bath medium.

### *Isolated cardiac preparations*

Guinea-pig heats were removed, dissected, and mounted in an isolated apparatus as described previously (Feigen *et al.*, 1960) under 1 g tension. Records of cardiac contractions were obtained with a tension transducer. Preparations were perfused with Chenoweth's solution (37°C) under 70 cm$H_2O$ pressure. The perfusate was collected, flow-rate recorded and the fluid bioassayed for ileal spasmogen.

Hearts were exposed to 026:B6(B) endotoxin at a concentration of 47 µg/ml in the perfusate. After initial challenge, 3 ml of Schedule B immune serum was recycled four times through the perfusion system, then the exposure to endotoxin was repeated.

### *Bioassay techniques*

Segments were exposed to standard bath concentrations of histamine to determine dose-response characteristics. All responses to other agents were expressed in terms of histamine equivalents according to the methods of Feigen *et al.* (1960).

### *Drugs*

The following drugs were used: histamine acid phosphate, mepyramine maleate, carbamyl chloride and semicarbazide.

## Results

### *Toxicity testing*

There were no changes detected in the condition of 6 animals injected with 0.5 mg/kg 026:B6(B) by either of the two routes of administration. However, 13/13 animals injected with 2 mg/kg of this endotoxin showed increased respiratory rate and decreased spontaneous movement at one hour. Four hours after injection the animals appeared drowsy and did not show startle responses. Deaths (4/13) occurred

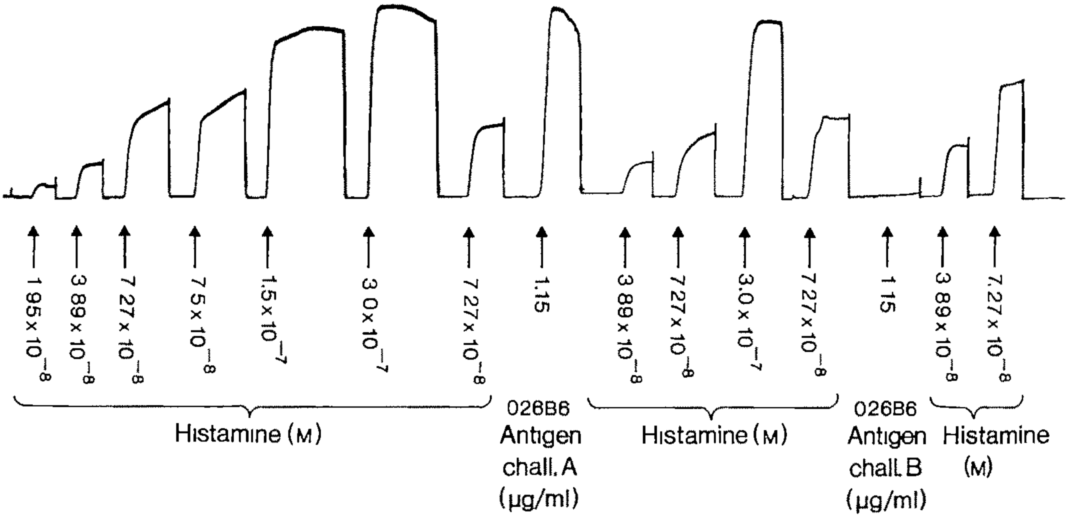

**Figure 1** Records of ileal contractions (isotonic lever). The ileum was removed from an animal actively immunized according to Schedule A against an endotoxic extract of *E. coli* 026:B6 cells (See text). Doses of endotoxin and histamine were applied as shown

between 8 and 36 h after injection. Before death, animals were lying prone with their eyes closed, breathing rapidly and shallowly and they were unresponsive to touch. There was no difference in the pattern of response to intraperitoneal injection when compared to intravenous injection.

### *Responses of isolated ilea to endotoxin*

Segments of ileum from 10 normal animals showed no response to the addition of 026:B6(B) endotoxin to the bath in concentrations up to 250 µg/ml.

In contrast all ileal segments ($n = 10$) from 5 animals immunized according to Schedule A showed contractile responses when 026:B6(B) was added to the bath over a test range defined by screening studies (see Methods section). The result of one experiment is illustrated in Figure 1. In the experiment shown, the initial exposure to endotoxin caused a contracture which terminated on washout of the bath. Repeat application of the same concentration of endotoxin caused a diminished response. The maximal responses to endotoxin in these experiments were $1.3 \pm 1.5 \times 10^{-8}$ M histamine equivalents with a range of $9 \times 10^{-9}$ to $3.8 \times 10^{-8}$ M histamine equivalents. Similarly endotoxin elicited contractile responses in ilea obtained from 6 animals immunized according to Schedule B. The responses were dose-dependent, showing increasing responses up to an optimal concentration. The maximal responses obtained were $6.4 \times 10^{-8} \pm 1.09 \times 10^{-7}$ M histamine equivalents with a range of 0 to $2.75 \times 10^{-7}$ M histamine equivalents.

### *Influence of passive transfer of immune serum on reactivity of ilea*

Three normal guinea-pigs were injected intraperitoneally with Schedule A immune serum 16 h before study of ileal segments. Reactivity to endotoxin was demonstrated in all ileal segments studied ($n = 7$). The properties of the reactions included dependence of the reaction on endotoxin concentration and desensitization as observed with actively immunized tissues.

A similar attempt was made to demonstrate passive transfer of reactivity to ileum together with transfer of Schedule B serum using five normal animals. Contractions were elicited in ilea from two animals; however, these responses could not be quantitated meaningfully as they were near to threshold for detection by this technique.

### *Influence of passive transfer of immune serum on reactivity of isolated hearts*

The hearts were exposed to 026:B6(B) at a concentration of 47 µg/ml in the perfusate. Initial challenge caused no change in the rate, rhythm, or strength of contractions of the isolated hearts, and no change in the rate of perfusate outflow. Challenge with the endotoxin was repeated after *in vitro* exposure to Schedule B immune serum (see Methods section). The results obtained with one heart are illustrated in Figure 2. The exposure caused disorganization of cardiac rhythm with atrioventricular dissociation

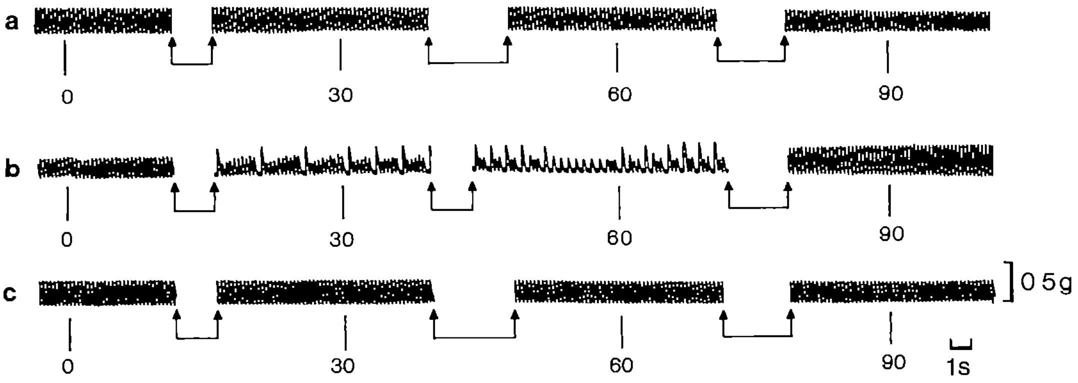

**Figure 2** Records of spontaneous contractions of an isolated preparation of guinea-pig heart recorded with a strain gauge tension transducer and polygraph recorder. Panels (a) to (c) show the effects of exposure to 47 µg/ml of 026:B6 (B) endotoxin under different conditions (a) Shows representative activity under control conditions at 0 time, 30, 60 and 90 s after the beginning of exposure to endotoxin; (b) shows the responses after passive transfer of immune serum (see text). In (c) the effects of a third application of endotoxin are shown. The antigen concentration was maintained in the perfusate for 60 s before return to toxin-free Chenoweth's solution. The symbols ↿⎯⎯↾ represent discontinuity in the traces occasioned by the paper speeds used (see time calibration).

(Figure 2). The perfusion rate fell from 5.2 ml/min to 1.6 ml/min and bioassay of the perfusate showed release of ileal spasmogen at a peak rate of $61.44 \times 10^{-8}$ M histamine equivalents/minute. Further challenge with endotoxin failed to produce any change in mechanical activity or perfusion rate, and there was no evidence of release of ileal spasmogen. Similar findings were recorded in experiments on two other normal hearts. The maximal release rates of ileal spasmogen in these experiments were 18 and $37.12 \times 10^{-8}$M histamine equivalents/minute.

### *Assay of bath fluid for reaction products*

After challenge of tissues with endotoxin, bathing media were collected, heated to 100°C for 30 min and then assayed on ilea from normal animals.

There was no evidence of release of ileal spasmogen by endotoxin from cardiac or ileal preparations from normal animals. In contrast, bath fluid taken after challenge of sensitized tissues with endotoxin caused ileal contractions which were blocked by mepyramine in parallel with blockade of effects of equivalent doses of histamine. Responses to carbamyl chloride (carbachol) were little affected by the blocking doses of mepyramine, suggesting that the ileal spasmogen released was histamine.

## Discussion

The endotoxin used in this study was toxic to normal adult guinea-pigs, yet it did not elicit anaphylactic reactions in ileal or cardiac preparations from normal adult guinea-pigs. From these observations it may be concluded that endotoxin toxicity is not necessarily dependent on anaphylactic reactivity as has been suggested since the earliest study of the two phenomena (Sanarelli, 1924).

The studies on tissues from actively immunized animals showed dose-dependent responses of ilea to exposure to the test endotoxin. Further challenge with the same dose of antigen produced a greatly diminished response. This phenomenon is termed desensitization, and is one of the characteristic features of the anaphylactic process, rather than being specific to any one antigen (Mongar & Schild, 1962). The tissue responses were paralleled by the release of histamine-like ileal spasmogens into the bath medium. Ileal and cardiac reactivity to endotoxin was shown to be transferred with transfer of immune serum. These observations suggest that endotoxins may elicit anaphylactic reactions in immunized animals, raising the possibility that this process might potentiate toxicity in such animals.

The reason for the lack of anaphylactic reactivity to 026:B6(B) in tissues of normal adult guinea-pigs remains unclear but may reflect a general lack of natural exposure to *E. coli* previously reported in these animals (Smith & Crabb, 1961; Smith, 1965) and confirmed for the colony used in these experiments (Boquest & McLean, unpublished observations).

Serological studies have provided evidence that most animals are naturally immunized against endotoxins. These findings possibly reflect the colonization of the bowel of most species by gram-negative organisms, including *E. coli* (Smith & Crabb, 1961). The demonstration that 026:B6(B) endotoxin may elicit anaphylactic reactions in immunized animals might have broad implications for the pathogenesis of endotoxin toxicity in most mammals.

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# COMPARISON OF THE EFFECTS OF CAFFEINE AND A 2-ALKYL-1,2,3-BENZOTRIAZINIUM IODIDE ON FROG RECTUS ABDOMINIS

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**1** The mode of action of 2-*n*-propyl-4-*p*-tolylamino-1,2,3-benzotriazinium iodide (TnPBI), which induced contractures of frog rectus abdominis muscle, was investigated.

**2** TnPBI caused contractures of frog rectus abdominis when the muscle had been depolarized with potassium chloride.

**3** Experiments with TnPBI and caffeine in calcium-free Ringer suggested that both compounds produce contractures by releasing intracellular bound calcium.

**4** It is suggested that at least two calcium stores are involved, one of which is sensitive to caffeine while both are sensitive to TnPBI.

## Introduction

Cull & Scott (1973) described the contractile effects on the frog rectus abdominis and chick biventer cervicis muscles of four series of 2-alkyl-1,2,3-benzotriazinium iodides. The mode of action of these compounds did not appear to involve acetylcholine receptors, since (+)-tubocurarine did not antagonize the contractures. Since quinidine and quinine produce similar effects, it was suggested that a common mode of action is involved. More recent work has suggested that the benzotriaziniums may produce their contractile effects by interfering with intracellular calcium binding sites in a manner similar to quinidine (Bondani & Karler, 1970) or caffeine (Axelsson & Thesleff, 1958).

This paper suggests that 2-*n*-propyl-4-*p*-tolylamino-1,2,3-benzotriazinium iodide (TnPBI, chosen as a representative of the benzotriaziniums) owes its contractile effects on the frog rectus to displacement of calcium ions from one or, more probably, two sites of intracellular binding.

## Methods

The rectus abdominis muscle from *Rana pipiens* was used in all experiments. The muscles were divided longitudinally and each half was mounted separately, under a resting tension of 0.5 g in a solution of the following composition (mM): NaCl 128, KCl 1.85, $CaCl_2$ 1.1, $NaHCO_3$ 11.9, $NaH_2PO_4$ 0.108 ('Starling Ringer') at room temperature (20–22°C) in a 10 ml bath gassed with atmospheric air. To assist relaxation after induced contractures, an additional tension of 1.0 g was applied to the tissue and removed 2 min before the next drug addition. The calcium-free Ringer solution was identical with Starling Ringer solution except for the absence of calcium chloride, and the addition of 0.1 mM ethylene-glycol-bis (*β*-aminoethyl-ether)-*N*,*N*$^1$-tetraacetic acid. Depolarizing Ringer solution was identical with Starling Ringer except that 128 mM sodium chloride was replaced by 128 mM potassium chloride. Contractures of the muscles were recorded on Washington Oscillographs, model 400 MD/2, using isotonic displacement transducers made in the section by Mr A. McK. French. In all experiments (except those performed in the presence of depolarizing Ringer) the tissues were initially set up in Starling Ringer and control contractures obtained to TnPBI (0.1 mM) and caffeine (4.0 mM). The Starling Ringer was then replaced by calcium-free Ringer (in which the tissues remained for the rest of the experiment), and the tissues left for 30 min, during which time they were washed several times with the calcium-free Ringer. Preliminary experiments showed that the muscles contracted slowly on exposure to calcium-free Ringer, and that these contractures reached a maximum within 15 to 20 minutes. When the additional tension was then applied, the tissues returned to their initial resting length within 5 minutes. In all further experiments, therefore, the additional tension was applied immediately after exposure to calcium-free Ringer, and the tissues were left under tension for 30 min, by which time all the tissues had relaxed to their original length.

Initial experiments showed that repeated additions of TnPBI (0.1 mM) to the tissues in calcium-free Ringer resulted in a progressive reduction of the contractile response until no response could be produced on further drug additions. Repeated additions of caffeine (4 mM) also resulted in progressive reduction to zero of the contractile responses as would be expected due to its calcium releasing properties. Four different procedures were then carried out:

(1) Control responses were obtained in Starling Ringer to 0.1 mM TnPBI. After the tissues had relaxed to their resting length under the additional tension, the Starling Ringer was replaced with depolarizing Ringer, in which the tissues remained for the rest of the experiment. This procedure resulted in a slowly developing contracture: under the influence of the additional tension, the tissues slowly relaxed to their original length after approximately 2 h: at this point another dose of 0.1 mM TnPBI was added.

(2) After obtaining control responses to 0.1 mM TnPBI and 4 mM caffeine in Starling Ringer, the tissues were bathed in calcium-free Ringer for the remainder of the experiment. The tissues were then dosed repeatedly with 4 mM caffeine until no further responses could be elicited. One dose of 0.1 mM TnPBI was then added to the tissues.

(3) In these experiments, after the control responses to TnPBI and caffeine in Starling Ringer had been obtained, the tissues were bathed in calcium-free Ringer (as in (2) above) and repeated doses of 0.1 mM TnPBI added until no further response could be elicited. One dose of 4 mM caffeine was then added to the tissues.

(4) Finally the effect, in calcium-free Ringer, of a single dose of TnPBI on a following dose of either TnPBI or caffeine, and the effect of a single dose of caffeine on a subsequent dose of TnPBI or caffeine was investigated.

Due to the differences in the time course of the action of TnPBI and caffeine, different time cycles were employed as follows: for TnPBI, a 10 min drug contact time every 45 min; for caffeine, a 5 min drug contact time every 15 minutes.

## Results

### *Procedure 1*

There was no significant difference between the contractile responses of the rectus muscle to 0.1 mM TnPBI in normal and depolarizing Ringer: the response of the depolarized tissue was 108.7% (s.e. 15.2, $n=9$) of the control responses. This suggests that membrane depolarization is not the mechanism by which TnPBI initiates the contractile process of this muscle.

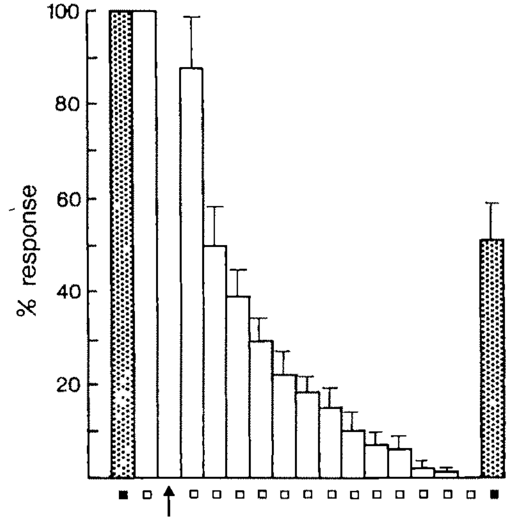

**Figure 1** The effect of repeated doses of caffeine 4 mM (□) to the frog rectus abdominis in calcium-free Ringer solution. The first two columns represent the control responses in Starling Ringer to 2-*n*-propyl-4-*p*-tolylamino-1,2,3-benzotriazinium iodide (TnBPI) 0.1 mM (stippled column, ■) and to caffeine 4 mM (open column, □). All other columns represent the mean responses (with s.e. mean represented by vertical bars) of six tissues, each individual value being calculated as a percentage of its own control response. The final testing dose of TnPBI was added when responses to caffeine had been abolished. Starling Ringer replaced by calcium-free Ringer at arrow.

### *Procedure 2*

Figure 1 illustrates the control responses to TnPBI and caffeine in Starling Ringer, and the decline to zero of the responses to caffeine on repeated dosage in calcium-free Ringer. The testing doses of 0.1 mM TnPBI produced responses which averaged 51.1% (s.e. 8.3, $n=6$) of the TnPBI control responses. This value is not significantly different from the first response of the tissue to 0.1 mM TnPBI (51.6%, s.e. 7.8, $n=6$) in calcium-free Ringer.

### *Procedure 3*

Figure 2 illustrates mean control responses to TnPBI and caffeine as in Figure 1, and the decline to zero of the responses to repeated doses of 0.1 mM TnPBI in calcium-free Ringer. The response to the testing dose of 4 mM caffeine was totally abolished in every experiment ($n=6$).

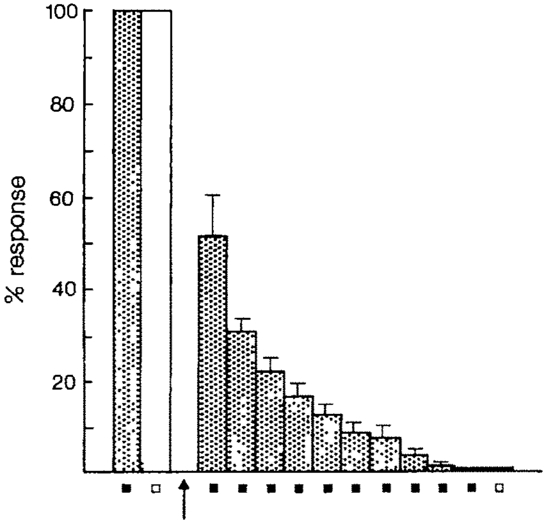

**Figure 2** The effect of repeated doses of 2-*n*-propyl-4-*p*-tolylamino-1,2,3-benzotriazinium iodide (TnPBI) 0.1 mM to the frog rectus abdominis in calcium-free Ringer solution. Control columns shading and symbols as in Figure 1. The values are also the mean of results from six tissues: the final testing dose of caffeine produced no response after responses to TnPBI had declined to zero.

Procedure 4

The administration of one dose of TnPBI had a greater depressant effect on a subsequent dose of TnPBI than it did on a subsequent dose of caffeine. In ten experiments, the response to a subsequent dose of TnPBI was reduced to 25.5% (s.e. 3.4) of the control value, while in four experiments, the response to a subsequent dose of caffeine was 51.3% (s.e. 8.9). These values are significantly different ($P < 0.01$). The addition of one dose of caffeine reduced the effect of a subsequent dose of caffeine to 48.6% (s.e 9.0, $n = 6$). This is not significantly different from the effect of a single dose of TnPBI on a subsequent dose of caffeine (51.3% as above). However, one dose of caffeine did not depress a following dose of TnPBI at all, the mean responses being 62.5% (s.e. 2.8, $n = 4$) of the control value. This is not significantly different from the initial response to TnPBI in calcium-free Ringer (51.6%, s.e. 7.8, $n = 6$), nor is it significantly different from the TnPBI responses obtained with procedure 2 after repeated caffeine dosage had resulted in complete extinction of the responses: the responses to the testing dose of TnPBI in this case being 51.1% (s.e. 8.3, $n = 6$).

## Discussion

Contractions of muscle tissue appear to be brought about by an increase in the free calcium ion concentration in the sarcoplasm (Heilbrunn & Wiercinski, 1947; Davis, 1963). When an isolated skeletal muscle is placed in a calcium-free medium, contractions may be elicited by compounds which release calcium ions from stores within the muscle. Caffeine (Frank, 1962), quinine (Benoit, Carpeni & Przybyslawski, 1964), and quazodine (Nott & Winslow, 1973) are examples of drugs which appear to cause contraction by a direct releasing action on calcium stores without causing any significant membrane depolarization. It seems probable that TnPBI causes muscle contractions in the frog rectus via a direct releasing effect on calcium stores, since potassium-depolarized tissue contracts in the presence of TnPBI, and also since contractions can be produced in both frog and chick biventer muscle in the presence of high concentrations of tubocurarine (Cull & Scott, 1973).

From the results of procedures 2 and 3, it may be concluded that TnPBI does release intracellular calcium, but that its mechanism of action is not identical to that of caffeine. There are two possible explanations of these results: firstly that only one calcium store is involved, and that, at the concentrations used, TnPBI is more effective than caffeine at releasing calcium ions, and so can release a greater percentage of this store than caffeine. This would enable TnPBI to produce contractions in calcium-free Ringer when caffeine is no longer effective. The alternative, more probable suggestion is that at least two sites of calcium storage exist and that TnPBI can release calcium from both stores while caffeine can release calcium ions from only one of them. It would appear from the results of procedure 4 that TnPBI is not more effective at releasing calcium ions from a single calcium store than is caffeine, since caffeine-induced responses are less affected than TnPBI-induced responses by the administration of a prior dose of TnPBI.

The second hypothesis of two binding stores therefore seems more probable, and suggests that TnPBI releases calcium from both stores while caffeine releases calcium from only one. Also continued dosage with caffeine had no significant effect on the response to a testing dose of TnPBI even when caffeine itself was no longer capable of eliciting a contracture (procedure 2). Benoit *et al.* (1964) reported that quinine could still produce a contracture of frog skeletal muscle in calcium-free Ringer when contractures to caffeine had been abolished by repeated administration of the latter. Batra (1974) has shown that two calcium stores exist, one associated with mitochondria and the other with sarcoplasmic reticulum. Quinidine preferentially released calcium from mitochondria while caffeine preferentially released calcium from fractionated sarcoplasmic reticulum. It would be interesting to investigate the effects of TnPBI upon these cell fractions.

We are grateful to Professor J.C. Gilbert for valuable discussion and suggestions during the preparation of this paper, and to Dr M.F.G. Stevens for the supply of TnPBI. C.K.M. was an S.R.C. scholar.

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# EFFECTS OF GUANETHIDINE ON HISTAMINE RELEASE DURING REFLEX VASODILATATION IN THE DOG

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1 The effects of guanethidine pretreatment on the release of [$^{14}$C]-histamine during the reflex vasodilatation induced in the atropinized gracilis muscle by rapid intravenous administration of noradrenaline, were studied in dogs.

2 After guanethidine treatment the haemodynamic reflex response was completely abolished and no appreciable modification of [$^{14}$C]-histamine release from the gracilis muscle following intravenous noradrenaline was observed.

3 These results suggest the hypothesis that the withdrawal of the sympathetic discharge represents the mechanism of histamine release during the reflex vasodilatation. Therefore, guanethidine would suppress both the passive and the histaminergic component of the baroreceptor reflex through the abolition of the sympathetic tone.

## Introduction

In previous work, designed to demonstrate the presence of afferents to vasodilator cholinergic encephalic centres in the sinus nerve of the dog, we observed that intravenous guanethidine administration reduced the vasodilatation in the perfused hind limb evoked by stimulation of the carotid sinus nerves and that intra-arterial atropine completely abolished the residual vasodilatation (Rengo, Perez, Chiariello, De Caprio, Saccà, Trimarco & Condorelli, 1976).

Based on previous observations suggesting that histamine participates as an active component in the genesis of reflex vasodilatation (Beck & Brody, 1961; Beck, 1965; Brody, 1966; 1968; Heitz, Shaffer & Brody, 1970), we interpreted the abolition of the reflex vasodilatation after treatment with guanethidine plus atropine as follows: while atropine blocks the cholinergic component alone, guanethidine not only abolishes the sympathetic vasoconstrictor tone but also blocks histamine release. Such an interpretation is also in agreement with the observation that reserpine, which shares with guanethidine a catecholamine depleting action, inhibits histamine release during reflex vasodilatation (Giotti, Guidotti, Mannaioni & Zilletti, 1966).

More recently, Weaver & Gebber (1974), recording electrical activity from the lumbar sympathetic nerve, immediately central to where section eliminated the active dilatation, observed only sympathetic inhibition in response to stimulation of the afferent vagus nerve or medullary depressor sites from which active dilatation had been produced. These authors, therefore, conclude that reflex vasodilatation does not result from excitation of any dilator pathway and cast doubt on the role of histamine in the genesis of the baroreceptor reflex. This interpretation would also be in agreement with a previous hypothesis of Glick, Wechsler & Epstein (1968), who suggested that antihistamines might reduce reflex vasodilatation by interfering with the reuptake of noradrenaline into adrenergic nerve terminals, rather than by antagonising the effects of histamine itself.

In the light of these data, the present work was mainly undertaken in order to establish whether guanethidine is really able to block the reflex vasodilatation by suppressing histamine release from the perfused atropinized gracilis muscle of the dog.

## Material and Methods

Experiments were performed on mongrel dogs of either sex, weighing 10–20 kg. Anaesthesia was induced with sodium thiopentone (Farmotal, Farmitalia) (30 mg/kg, i.v.); additional doses of 30–50 mg were given as required. The trachea was intubated and artificial ventilation was performed at a rate of 13–16 cycles per min after skeletal muscle relaxation induced by succinylcholine (Midarine, Wellcome) (0.2 mg/kg, i.v.). Body temperature was periodically measured and maintained between 37–37.5°C. The arterial $P\text{O}_2$ was maintained above 90 mmHg and $P\text{CO}_2$ below 35 mmHg; arterial pH was about 7.40. The arterial $P\text{O}_2$, $P\text{CO}_2$ and pH were measured by a blood gas analyzer (Corning EEL 165).

The perfused region was the gracilis muscle isolated according to the technique of Renkin & Rosel (1962). The perfusion was performed at constant flow with blood of the same animal by means of a peristaltic pump (Sigmamotor T6S). For this purpose after administration of heparin (Liquemin, Roche) (5 mg/kg, i.v.), a polyethylene catheter was introduced through the external iliac artery into the abdominal aorta and blood was reinfused, by the perfusion pump, into the gracilis main artery. Under such conditions, changes in vascular tone of the perfused region were reflected by proportional changes in perfusion pressure. Blood flow was adjusted to give a perfusion pressure approximately equal to the systemic blood pressure and was left unchanged throughout the course of the experiment. Perfusion pressure was measured through a T connector interposed between the pump and the perfused region, while arterial blood pressure was recorded by a catheter placed in the thoracic aorta via the contralateral femoral artery. A Battaglia-Rangoni multichannel polygraph and pressure transducers were used.

Endogenous substances released by systemic hypertension and arterial catecholamines were delayed in their arrival to the perfused gracilis muscle because of the long time required to pass through the pump and therefore did not exert any influence on the recording of the haemodynamic phenomena.

The vein of the gracilis muscle was cannulated and the effluent blood was collected into a beaker and was not returned to the experimental animal so that drugs administered intra-arterially to the gracilis muscle had only peripheral effects since they never reached the systemic circulation. Heparin-treated blood of another dog was infused into the contralateral femoral vein at the same rate as the blood flowing from the gracilis vein.

At least 20 min were allowed after the completion of all surgical procedures before beginning the experiment.

In five dogs baroreceptor stimulation was carried out by rapid intravenous injection of noradrenaline (2 μg/kg). Since the participation of the cholinergic system in the genesis of the reflex vasodilatation has been recently demonstrated (Takeuchi & Manning, 1971; 1973; Ellison & Zanchetti, 1973; Rengo, Chiariello, De Caprio, Saccà, Trimarco, Perez & Condorelli, 1975; Chiariello, Condorelli, De Caprio, Rengo, Saccà & Trimarco, 1976), the muscle was atropinized in order to abolish this vasodilator component. After the intra-arterial administration of atropine (0.3 mg), the completeness of the cholinergic blockade was tested by the abolition of the haemodynamic response to the intra-arterial injection of acetylcholine (0.5 μg); then, the first reflex response was evoked by intravenous noradrenaline administration. In the same way, after intravenous administration of guanethidine, the effectiveness of the adrenergic blockade was first ascertained by the lack of change in systemic and perfusion pressure following the occlusion of the carotid arteries, and then a second response to the intravenous injection of noradrenaline was also evaluated. Responses were elicited at 20 min intervals.

To confirm the specificity of the pharmacological blockade of atropine and guanethidine, the haemodynamic response to intra-arterial administration of noradrenaline (0.1 μg) and sodium nitrite (0.15 mg), before and after administration of atropine and atropine plus guanethidine was evaluated in five experiments.

In order to investigate whether both reflex vasodilatation and the increase of histamine release induced by the intravenous injection of noradrenaline could be ascribed to baroreceptor stimulation, five experiments were performed in which the reflex was evoked before and after surgical selective baroreceptor denervation.

In the experiments in which the release of [$^{14}$C]-histamine was evaluated from the gracilis muscle during the baroreceptor reflex, 25 μCi of histamine [2 ring-$^{14}$C]-dihydrochloride (specific activity 59 mCi/mM, Amersham) in 1 ml of 0.9% w/v NaCl solution (saline) was infused into the perfusion tubing over a 5 min period. The radioactive venous effluent, collected only during the infusion of radiolabelled histamine, was reperfused into the gracilis muscle through a side arm on the inflow side of the Sigmamotor pump. A 40 min period was allowed for the radioactive material in the venous effluent to reach a stable level (Brody, 1966). All samples were collected in chilled tubes containing 6 ml of 0.6 N $HClO_4$.

Preresponse samples were collected in each case to determine the basal levels of radioactive materials in the blood. During the various responses studied, 30 s samples were collected continuously for a total of 2 minutes.

Total radioactivity was measured by mixing an aliquot of the perchloric acid extract with 10 ml of Instagel (Packard Instrument). [$^{14}$C]-histamine was extracted according to the method of Snyder, Axelrod & Bauer (1964). The method used in this study give a fairly accurate measurement of histamine output, as shown by Brody (1966).

To exclude the possibility that time-dependent modifications in the increase of [$^{14}$C]-histamine release

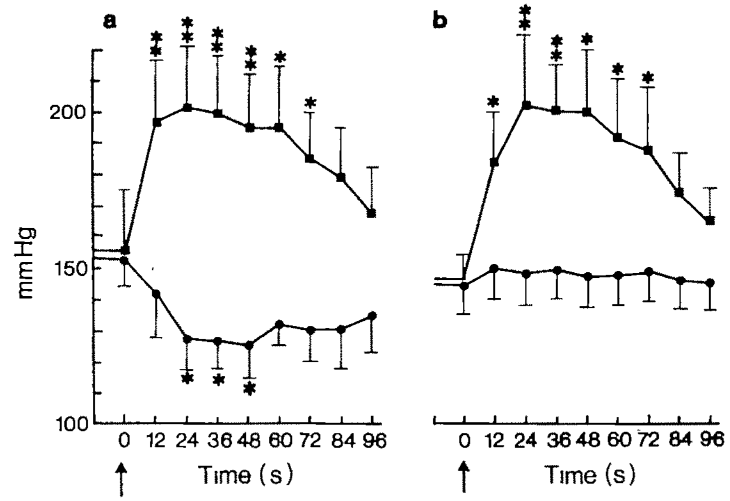

**Figure 1** Effects of intravenous injection of noradrenaline before (a) and after (b) intravenous administration of guanethidine: (■) blood pressure; (●) perfusion pressure. Each point represents mean of 5 observations. Vertical lines show s.e. mean. Arrows indicate the time of noradrenaline injection. Statistical comparison is made between 0 time and each point using *t* test for paired samples.
$^*P<0.05$; $^{**}P<0.01$.

during the vasodilator response might occur, five experiments were performed in which two reflexes were evoked by intravenous injection of noradrenaline (2 µg/kg) at 30 min intervals without any pharmacological treatment.

The following drugs were used: atropine (Lancellotti); guanethidine (Ismelin, Ciba) (8 mg/kg, i.v.); acetylcholine (Koch-Light) (0.5 µg, i.a.); noradrenaline (Noradrec, Recordati).

Statistical analysis was conducted by standard techniques (Snedecor & Cochran, 1967).

## Results

The intravenous noradrenaline injection induced a systemic hypertension and a statistically significant decrease of perfusion pressure in the atropinized gracilis muscle circulation (Figure 1a). After guanethidine treatment, the intravenous injection of noradrenaline did not induce any haemodynamic change in the perfused gracilis, while the systemic hypertensive response was unmodified (Figure 1b).

On the other hand, neither atropine nor atropine plus guanethidine modified the response to the intra-arterial administration of noradrenaline and sodium nitrite (Table 1).

The results of the experiments with labelled histamine are summarized in Figure 2. It is clear that during the reflex vasodilatation secondary to intravenous noradrenaline, both total radioactivity and [$^{14}$C]-histamine significantly increased in the blood flowing from the gracilis muscle. After guanethidine treatment, no appreciable modification of [$^{14}$C]-histamine release was observed. Moreover, the comparison between the release of [$^{14}$C]-histamine due to reflex vasodilatation at corresponding times before and after guanethidine administration, shows that there is a significant difference between the release rates (Figure 2).

On the other hand, in the experiments in which the reflex was evoked at 30 min intervals without any pharmacological treatment, no statistically significant change in the increase of [$^{14}$C]-histamine release during the reflex responses could be demonstrated by covariance analysis (Table 2).

The results of the experiments performed to establish the selectiveness of the baroreceptor stimulation indicate that the selective baroreceptor denervation is able to abolish both the dilatation and the increase in histamine release (Table 3).

## Discussion

The observation that intravenous guanethidine administration completely abolishes the reflex vasodilatation confirms our previous results regarding the abolition of the reflex response to the electrical stimulation of the sinus nerves in atropinized hind limb (Rengo *et al.*, 1976).

On the other hand, the finding that neither atropine nor guanethidine induced any modification of the haemodynamic response to the intra-arterial injection

**Table 1** Effects of intra-arterial noradrenaline and sodium nitrite on blood pressure and perfusion pressure in gracilis of dog

| | | Seconds after drug injection | | |
|---|---|---|---|---|
| Treatment | | 0 | 36 | 72 |
| Noradrenaline alone | BP | 186±13 | 186±13 | 186±13 |
| | PP | 199±8 | 228±9<br>$P<0.005$ | 213±9<br>$P<0.05$ |
| After atropine | BP | 186±13 | 186±13 | 186±13 |
| | PP | 198±8 | 229±9<br>$P<0.001$ | 214±9<br>$P<0.025$ |
| After atropine plus guanethidine | BP | 186±13 | 186±13 | 186±13 |
| | PP | 211±8 | 235±10<br>$P<0.01$ | 222±10<br>$P<0.05$ |
| Sodium nitrite alone | BP | 178±3 | 178±3 | 178±3 |
| | PP | 189±11 | 150±10<br>$P<0.01$ | 180±12<br>$P<0.02$ |
| After atropine | BP | 178±3 | 178±3 | 178±3 |
| | PP | 188±11 | 154±11<br>$P<0.01$ | 179±12<br>$P<0.05$ |
| After atropine plus guanethidine | BP | 178±3 | 178±3 | 178±3 |
| | PP | 188±12 | 149±9<br>$P<0$ 005 | 176±12<br>$P<0.05$ |

Each value represents mean ± s.e. of results from five animals; it is compared with the corresponding value at time 0 by *t* test for paired samples. BP = blood pressure; PP = perfusion pressure in mmHg.

of noradrenaline and sodium nitrite, proves that the vascular system was reactive following these pharmacological blockades. Furthermore, that the blockade of adrenergic discharge induced by guanethidine treatment was genuinely effective is demonstrated by the lack of change in perfusion and systemic pressure following carotid artery occlusion. We have already demonstrated the capacity of guanethidine to block the sympathetic discharge in the dog (Rengo, De Caprio, Saccà, Trimarco, Perez, Chiariello & Condorelli, 1975a). In this animal, guanethidine is able to reverse the vasoconstriction induced by stimulation of the lumbar sympathetic chain to vasodilatation.

Our results, obtained with the use of [$^{14}$C]-histamine, confirm the results of Tuttle (1967) and Brody (1968) that radioactive histamine release from

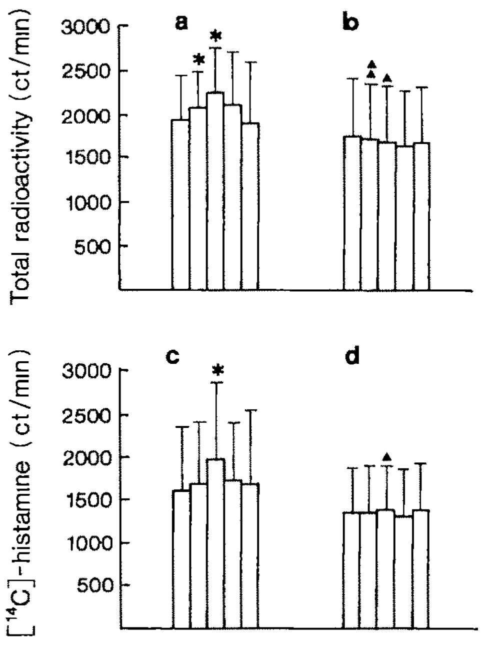

**Figure 2** Changes in total and [$^{14}$C]-histamine radioactivity of the venous blood effluent from the perfused gracilis following the intravenous administration of noradrenaline in basal state (a and c) and after intravenous administration of guanethidine (b and d). Each column shows the radioactivity of venous blood samples collected for 30 s; the first before, the others after the beginning of the reflex vasodilatation. $n=5$.

*$P<0.05$ when statistical comparison is made between base and each bar using *t* test for paired samples.

▲$P<0.05$ and ▲▲$P<0.01$ when statistical comparison is made between corresponding times before and after intravenous administration of guanethidine by covariance analysis.

**Table 2** Changes in total radioactivity (ct/min) of the venous blood effluent from the perfused gracilis muscle of the dog following two intravenous injections of noradrenaline at 30 min intervals

| *Samples* | *1* | *2* | *3* | *4* | *5* |
|---|---|---|---|---|---|
| First noradrenaline injection | 1591±494<br>NS | 1969±536<br>NS | 1795±473<br>NS | 1609±490<br>NS | 1438±419<br>NS |
| Second noradrenaline injection | 1273±371 | 1603±496 | 1591±382 | 1413±400 | 1120±320 |

Each value represents mean±s.e. of five experiments. Each sample is collected for 30 s; the first sample before the drug injection, the others after the beginning of the reflex vasodilatation. Statistical comparison is made between corresponding samples of the two stimulations using covariance analysis.

**Table 3** Effects of intravenous noradrenaline on (A) systemic and perfusion pressure and (B) total radioactivity (ct/min) of the venous blood effluent from the perfused gracilis muscle before and after selective baroreceptor denervation

| | | | *Seconds after drug injection* | | |
|---|---|---|---|---|---|
| A | *Treatment* | | *0* | *36* | *72* |
| | Noradrenaline alone | BP | 90±8 | 104±9<br>$P<0.05$ | 94±12<br>NS |
| | | PP | 111±15 | 89±15<br>$P<0.001$ | 111±15<br>NS |
| | After selective baroreceptor denervation | BP | 93±10 | 110±8<br>$P<0.005$ | 90±7<br>NS |
| | | PP | 99±3 | 95±4<br>NS | 91±8<br>NS |

| | | | | | | |
|---|---|---|---|---|---|---|
| B | *Samples* | *1* | *2* | *3* | *4* | *5* |
| | Noradrenaline alone | 1050±147 | 1868±110<br>$P<0.01$ | 1492±72<br>$P<0.01$ | 1214±82<br>NS | 1233±137<br>NS |
| | After selective baroreceptor denervation | 930±78 | 924±72<br>NS | 916±58<br>NS | 938±72<br>NS | 941±83<br>NS |

BP=blood pressure; PP=perfusion pressure in mmHg.
Each value represents mean±s.e. of five experiments. Each sample is collected for 30 s, the first before, the others after the drug injection.
Statistical comparison is made between values at 0 time and each following time using the paired *t* test.

the gracilis muscle increases during reflex vasodilatation. The complete disappearance of the histaminergic component after guanethidine treatment suggests that guanethidine not only inhibits the sympathetic discharge but also abolishes the histaminergic component. This result is of interest because the ability to prevent histamine release has been previously observed only for α-adrenoceptor blocking agents such as phentolamine (Boerth, Ryan & Brody, 1970) and phenoxybenzamine (Heitz & Brody, 1975).

The mechanism underlying histamine release during reflex vasodilatation is not completely clear, Ryan & Brody (1972), in the light of the results obtained by the chronic denervation of the dog gracilis muscle, suggested that either the postganglionic adrenergic fibres innervate the vascular smooth muscle as well as a separate non neural cell from which histamine could be liberated, or that two separate adrenergic fibres exist: one innervating vascular smooth muscle causing vasoconstriction, and a second innervating a histamine containing cell. These hypotheses would be in agreement with the observation of Graham & Lioy (1973) who demonstrated the presence in the lumbar ventral roots of the preganglionic segment of a histaminergic pathway affecting vascular resistance in the hind limb of the dog. These authors suggest, therefore, that the preganglionic segment of this histaminergic pathway runs isolated in the ventral roots from L5 to L7; the postganglionic fibres would then presumably pass into the sympathetic chain together with the adrenergic and cholinergic fibres. Such a view has been also confirmed by recent studies of Heitz & Brody (1975) on the genesis of the vasodilatation occurring when stimulation of the distal ending of the cut sympathetic chain was terminated.

However, the observation that guanethidine-treatment abolishes histamine release during reflex vasodilatation in the perfused region certainly does not elucidate definitely the mechanisms underlying this phenomenon. In accordance with the hypothesis of Beck, Pollard, Spalding & Wise (1971) that the tonic release of noradrenaline would stabilize the membrane of the histamine containing cell and prevent the liberation of histamine, it might be suggested that the enhanced release of histamine during reflex vasodilatation depends on the withdrawal of the sympathetic discharge. Therefore, guanethidine would supress both the passive component of the reflex vasodilatation and at the same time the release of histamine through the same mechanism, consisting of the abolition of the sympathetic vasoconstrictor tone. Such an interpretation would also be in agreement with the results of Weaver & Gebber (1974) about the lack of excitation of a dilator pathway, running in the sympathetic chain, during reflex vasodilatation. In fact, according to Beck *et al.*'s definition (1971), the histaminergic component of this haemodynamic phenomenon should be considered active only because it takes place through the release of a vasodilator substance and not on account of the electrical activation of a vasodilator nervous pathway.

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# COMPARATIVE EFFECTS OF HYPOPHYSECTOMY AND ADRENALECTOMY UPON PLASMA AND ADRENAL MONOAMINES IN PREGNANT AND NON-PREGNANT RATS

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1 The influence of hypophysectomy and adrenalectomy upon release and storage of adrenaline and noradrenaline during rat oestrous cycle and pregnancy has been studied.

2 Hypophysectomy during the pro-oestrous phase of the oestrous cycle increased plasma noradrenaline concentration which was unchanged, however, by similar treatment in pregnant females. After adrenalectomy during dioestrous and pro-oestrous phases, as well as on day 15 post-coitum, plasma noradrenaline showed an increase, although the increase in pregnant females was greater.

3 Adrenocorticotrophic hormone (ACTH) administration to non-pregnant and pregnant females increased plasma adrenaline values significantly, compared with control values. Treatment of adrenalectomized rats with hydrocortisone lowered plasma noradrenaline to control values in both non-pregnant and pregnant females.

4 Hypophysectomy produced a decrease in plasma adrenaline content, whilst ACTH treatment increased it. Plasma adrenaline disappeared completely after adrenalectomy, during the pro-oestrous phase and pregnancy, but extirpation of adrenals during the dioestrous phase still left appreciable amounts of adrenaline in plasma. Hydrocortisone treatment of both non-pregnant and pregnant adrenalectomized females resulted in the reappearance of plasma adrenaline

5 Adrenal stores of adrenaline and noradrenaline declined after hypophysectomy but the decreases were greater in pregnant females.

6 Compensatory administration of ACTH to hypophysectomized females increased adrenaline and noradrenaline content of adrenals to control non-pregnant female values during the pro-oestrous phase of the oestrous cycle but similar treatment in pregnant females resulted in an increase in adrenaline.

7 Although ACTH administration increased adrenal noradrenaline levels in pregnant hypophysectomized females, the mean value remained below that of non-operated pregnant females.

8 The results suggest that, during oestrous cycle and pregnancy the effects of pituitary and adrenal hormonal deficiency upon storage and release of monoamines differ quantitatively.

## Introduction

Earlier studies suggest that the hormones of the adrenal cortex and the medullary monoamines act as a physiological unit (Britton, 1930; Ramey & Goldstein, 1957). Some of the physiological effects of adrenaline and noradrenaline do not occur in the absence of glucocorticoid hormones (Axelrod, 1963; 1965; Schayer, 1964). The isolation and characterization of the adrenal medullary enzyme, phenylethanolamine *N*-methyltransferase, provided a tool to study the possible link between adrenomedullary and adrenocortical function (Axelrod, 1962). It has been reported that adrenaline content and activity of this enzyme in adrenal gland are greatly decreased after hypophysectomy in adult animals (Wurtman & Axelrod, 1965; 1966). Further investigations suggest that glucocorticoids in adrenal portal blood are essential for the conversion of noradrenaline to adrenaline and for the activity of phenylethanolamine *N*-methyltransferase in the adrenal medulla (Wurtman, 1966; Parvez, Parvez & Youdim, 1975). Our recent studies also provide evidence to show that the rate of monoamine metabolism is reduced by a decrease in glucocorticoid concentration in the circulation (Parvez & Parvez, 1973a, b). Both monoamine oxidase and catechol *O*-

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methyltransferase activities are significantly higher in animals in which glucocorticoid production is blocked pharmacologically or surgically (Parvez & Parvez, 1972a, 1973c; Youdim & Holzbauer, 1976).

The present experiments were performed to compare the effects of hypophysectomy and adrenalectomy upon storage and release of adrenaline and noradrenaline between rats in the pro-oestrous phase of the oestrous cycle and pregnant rats. Plasma levels of adrenaline and noradrenaline were determined six days after hypophysectomy or adrenalectomy, during pro-oestrus or on day 15 post-coitum. The operated females also received compensatory administration of hydrocortisone or adrenocorticotrophic hormone (ACTH) to assess the effect of these two hormones during oestrous cycle and pregnancy.

## Methods

Albino female rats of Sherman strain, obtained from Janvier, Paris, and weighing 250 ± 50 g, were housed at a constant temperature of 21°C with natural day and night cycles. They were fed with commercial laboratory food *ad libitum*. The rats were impregnated by keeping one male with seven females in a cage from 18 h 00 min to 09 h 00 min once only. Fertilization was defined as having occurred at 02 h 00 min. Thus, it was possible to estimate an approximate time of pregnancy (±7 hours). The animals were palpated abdominally 14 days later to verify the pregnancy. If positive, at 02 h 00 min they were considered to be 14.5 days pregnant. Under normal conditions parturition took place between 21.5 and 22 days of pregnancy (Parvez *et al.*, 1975).

### *Hypophysectomy and ACTH administration*

Control female rats in pro-oestrus (non-pregnant) were identified by examining vaginal smears. Only those with well-defined symptoms of pro-oestrus were used. Pregnant females were used on the 15th day of gestation. Hypophysectomy was performed parapharyngealy under ether anaesthesia (Smith, 1926). Hypophysectomized animals were maintained on sucrose water together with normal laboratory food. All operated females were checked for effectiveness of operation after they were killed. Another group of non-pregnant and pregnant hypophysectomized females received daily injection of ACTH (1.3 iu/100 g body weight) from day 16 post-coitum to day 21 post-coitum. Hypophysectomy did not alter the time of parturition.

### *Adrenalectomy and hydrocortisone treatment*

The animals were adrenalectomized bilaterally, either on day 15 of pregnancy or during the well-defined pro-oestrous phase of the oestrous cycle. They were given salt water in addition to commercial laboratory food. Some animals in each group received daily injections of hydrocortisone (3 mg/100 g body weight) for 6 days.

### *Isolation of adrenals and plasma*

Five hours after the last injection the animals were killed by neck fracture and blood collected and adrenals excised within 1 minute. This procedure avoided shock. A maximum of 3 ml blood was taken from the abdominal aorta of each rat. Most samples of blood were free from haemolysis and only such plasma was used for catecholamine analysis.

### *Assay of adrenaline and noradrenaline in adrenals*

The adrenals were carefully cleaned of fat, weighed and homogenized in 2 ml of 4% (w/v) trichloroacetic acid at 0°C. Adrenal adrenaline and noradrenaline were isolated according to the technique of Euler & Lishajko (1961) using the trihydroxindole reaction for fluorimetric estimation.

### *Assay of plasma catecholamines*

Heparinized blood from 5 rats was pooled in a glass centrifuge tube containing 2 ml of potassium fluoride, 2% (w/v) and potassium thiosulphate, 3% (w/v). The blood was centrifuged at 4°C for 10 min at 4000 *g* and adrenaline and noradrenaline in plasma were isolated by adsorption on acid activated aluminium oxide (Euler & Lishajko, 1959). The batch process was preferred to column as recoveries were better (Anton & Sayre, 1962). One gram of alumina was washed twice with glass distilled water and the plasma added to a centrifuge tube. The pH was adjusted to 8.4 by glass electrode pH meter with continuous shaking for 4 minutes. The tubes were then centrifuged at 2000 *g* for 1.5 min and the supernatant discarded. The alumina was washed with 20 ml of distilled water and both adrenaline and noradrenaline were eluted with 4 ml of 0.25 N acetic acid. The trihydroxindole procedure was employed for simultaneous estimation of both amines.

All the results were subjected to statistical analysis by Fisher's *t* test. Mean values are expressed ± s.e. mean.

## Results

### *Plasma adrenaline after hypophysectomy and adrenalectomy*

Figure 1 shows the influence of pituitary ablation upon plasma adrenaline concentration in non-pregnant and pregnant females. After hypophysectomy, values in non-pregnant female rats decreased significantly, by

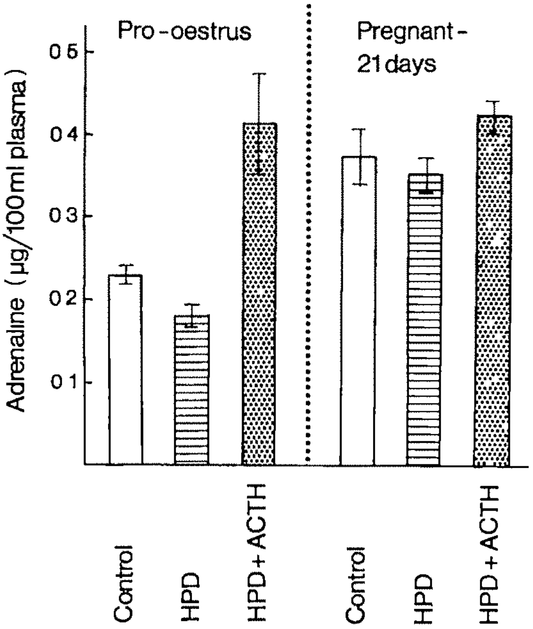

**Figure 1** Influence of hypophysectomy in female rats during pro-oestrous phase of the oestrous cycle or on 15th day post-coitum upon plasma adrenaline content. The animals were killed 6 days later. Hypophysectomized rats (HPD); Hypophysectomized rats receiving daily injections of ACTH (HPD + ACTH). Each group represents the mean value of at least 8 to 12 individual values. Vertical lines show s.e. mean. Statistical differences between the different groups are as follows:

*Pro-oestrus*

| | | |
|---|---|---|
| Control | vs HPD | $P<0.05$ |
| Control | vs HPD+ACTH | $P<0.01$ |
| HPD | vs HPD+ACTH | $P<0.001$ |

Pregnant 21 days

| | | |
|---|---|---|
| Control | vs HPD | NS |
| Control | vs HPD+ACTH | NS |
| HPD | vs HPD+ACTH | NS |

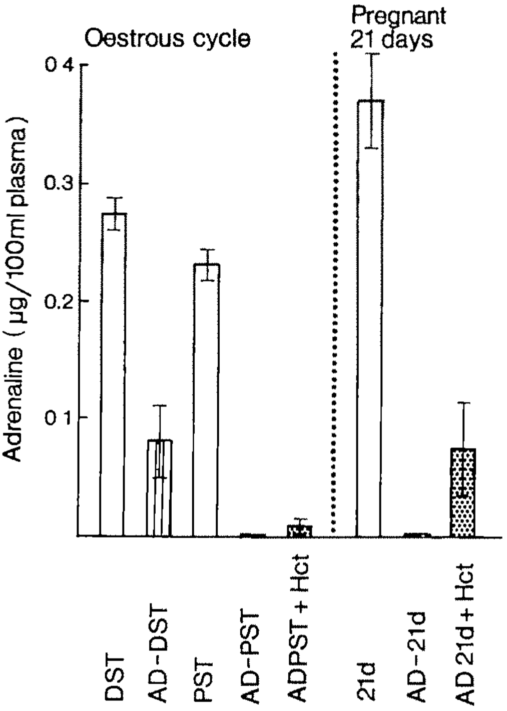

**Figure 2** Plasma adrenaline levels in female rats adrenalectomized during dioestrous or pro-oestrous phase of the oestrous cycle or on 15th day of pregnancy. The animals were killed 6 days after operation, some receiving hydrocortisone supplementation. The mean value for each group was calculated from at least 12 to 20 individual values. Dioestrus (DST); pro-oestrus (PST); adrenalectomized rat (AD); hydrocortisone (Hct); and adrenalectomized 21 day pregnant rats (AD21d). Statistical differences between groups are as follows:

*Oestrous cycle*

| | | |
|---|---|---|
| DST | vs AD−DST | $P<0.001$ |
| PST | vs AD−PST+Hct | $P<0.001$ |

*Pregnant–21 days*

| | | |
|---|---|---|
| AD–21d | vs AD−21d+Hct | $P<0.001$ |

20% compared with controls. The pregnant females showed a small but not significant decrease in plasma adrenaline concentration after pituitary ablation. Compensatory treatment with ACTH (1.3 iu/100 g body weight daily) for 6 days brought about a pronounced increase in hypophysectomized non-pregnant females but similar treament in hypophysectomized pregnant females produced no significant effect.

The effect of adrenalectomy on plasma adrenaline concentration in pregnant and non-pregnant rats is shown in Figure 2. Females adrenalectomized during pro-oestrus or on day 15 post-coitum manifested a complete absence of adrenaline in their plasma. Females adrenalectomized during dioestrus showed a relative rather than absolute decrease in plasma adrenaline. Treatment of adrenalectomized pro-oestrous females with hydrocortisone (3 mg/100 g body weight) for 6 days increased plasma adrenaline concentration. Similar hydrocortisone dosage in females adrenalectomized on day 15 of pregnancy produced a significant increase in plasma adrenaline level although the mean value remained well below that of controls.

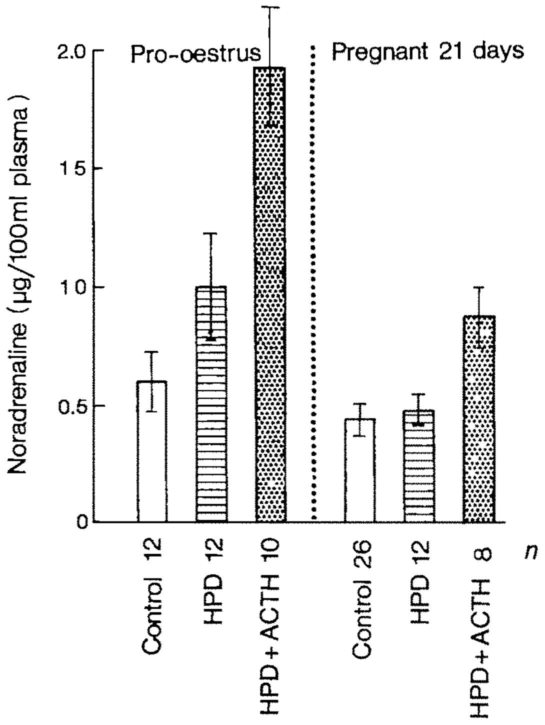

**Figure 3** Plasma noradrenaline levels in non-pregnant and pregnant female rats subject to hypophysectomy. Each group represents the mean value of at least 8 to 26 rats Hypophysectomized female rats receiving ACTH treatment daily (HPD).

Statistical differences between groups are as follows:

| | | |
|---|---|---|
| *Pro-oestrus* | | |
| Control | vs HPD | NS |
| HPD | vs HPD+ACTH | $P<0.01$ |
| *Pregnant 21d* | | |
| HPD | vs HPD+ACTH | $P<0.01$ |

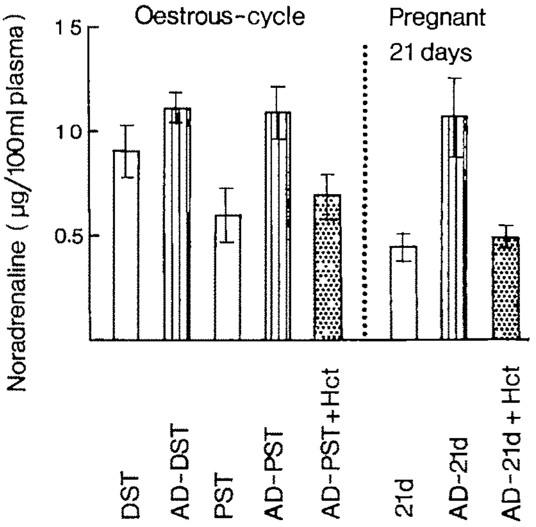

**Figure 4** Effect of adrenalectomy on plasma noradrenaline levels of non-pregnant female rats during di-oestrous (AD–DST) or pro-oestrous phase (AD–PST). The pregnant animals were adrenalectomized on day 15 post-coitum (AD–21d). similar groups also received daily injections of hydrocortisone (Hct). All animals were killed 6 days after operation, some receiving hydrocortisone supplementation. Each group represents the mean value of at least 10 to 26 rats. Vertical lines show s.e. mean.

Statistical differences between groups are as follows:

| | | |
|---|---|---|
| *Oestrous cycle* | | |
| PST | vs AD–PST | $P<0.02$ |
| AD–PST | vs AD–PST+Hct | $P<0.02$ |
| *Pregnant 21d* | | |
| 21d | vs AD–21d | $P<0.01$ |
| AD–21d | vs AD–21d+Hct | $P<0.01$ |

### *Plasma noradrenaline after hypophysectomy and adrenalectomy*

Figure 3 shows the effects of hypophysectomy on plasma noradrenaline concentration in pregnant and non-pregnant rats, 6 days after operation. Pituitary ablation during pro-oestrus resulted in a 40% increase in plasma value; similar treatment in pregnant rats, however, produced little change. Daily administration of ACTH to pro-oestrous hypophysectomized females resulted in a 93% increase of noradrenaline when compared with control operated females. Pregnant females treated with ACTH responded in a similar manner to non-pregnant females although the increase was somewhat less marked (80%).

Adrenalectomy in pregnant and non-pregnant females resulted in a highly significant increase in plasma noradrenaline concentration in the former (143% higher than the non-operated pregnant value, Figure 4). Adrenalectomy on oestrous rats had a similar qualitative effect but differed quantitatively from that in pregnant females. Adrenalectomy during dioestrus and pro-oestrus increased plasma noradrenaline concentration by 24 and 85% respectively, the latter being significantly different from its pro-oestrous control value. Replacement therapy of non-pregnant and pregnant adrenalectomized rats with hydrocortisone decreased plasma noradrenaline values, the means then being similar to those of non-operated controls (Figure 4).

### *Adrenal catecholamine content in non-pregnant and pregnant hypophysectomized rats*

Figure 5 compares adrenaline content of the adrenal glands of non-pregnant and pregnant females after

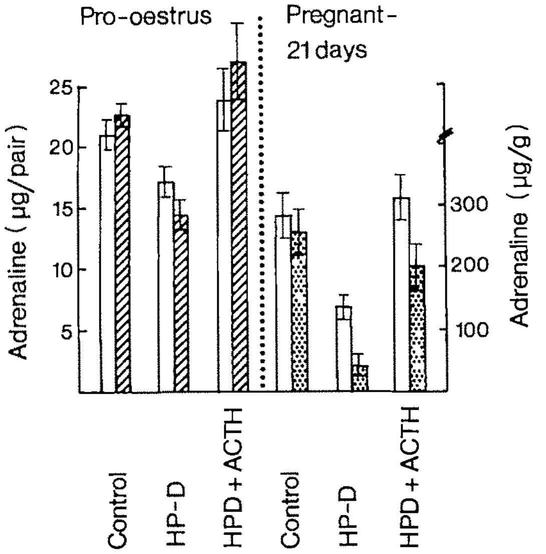

**Figure 5** Influence of hypophysectomy (HPD) on adrenal adrenaline content in female rats during pro-oestrous phase of the oestrous cycle or on 15th day of pregnancy. The animals were killed 6 days after operation. The hatched columns show values as μg/g of adrenal gland, the open columns as μg/pair of adrenals. Each group represents the mean of at least 10 to 26 rats. Vertical lines show s.e. mean.

Statistical differences are as follows:

*Pro-oestrus*

| | | μg/pair | μg/g |
|---|---|---|---|
| Control | vs HPD | $P<0.01$ | $P<0.005$ |
| HPD | vs HPD+ACTH | $P<0.02$ | $P<0.005$ |
| *Pregnant 21d* | | | |
| Control | vs HPD | $P<0.005$ | $P<0.001$ |
| HPD | vs HPD+ACTH | $P<0.01$ | $P<0\ 01$ |

After ACTH administration, mean values in non-pregnant and pregnant hypophysectomized females were not significantly different from control values.

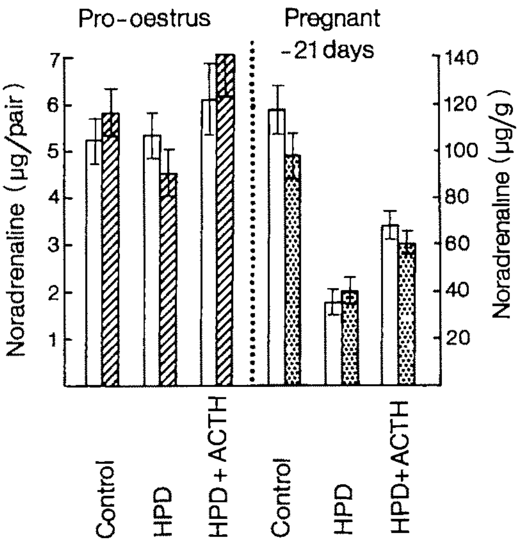

**Figure 6** Influence of hypophysectomy (HPD) on adrenal noradrenaline content in female rats during pro-oestrous phase of the oestrous cycle or on the 15th day of pregnancy. All the animals were killed 6 days after operation One group of each type also received ACTH throughout the post-operative period. Mean values are calculated from 10 to 26 individual rats. Vertical lines show s.e. mean.

The statistical differences between the groups are as follows:

*Pro-oestrus*

| | | μg/pair | μg/g |
|---|---|---|---|
| Control | vs HPD | NS | NS |
| HPD | vs HPD+ACTH | NS | $P<0.05$ |
| *Pregnant 21d* | | | |
| Control | vs HPD | $P<0.01$ | $P<0.01$ |
| HPD | vs HPD+ACTH | $P<0.01$ | $P<0.05$ |
| Control | vs HPD+ACTH | $P<0.01$ | $P<0.01$ |

hypophysectomy. The results are expressed both as μg/pair of glands and μg/g adrenal weight. After hypophysectomy, total adrenal adrenaline of pro-oestrous rats decreased by 19% and the decrease was more marked (38%) when expressed as μg/g. Pituitary ablation in pregnant females produced a significant decrease in adrenal adrenaline content (53% when expressed as μg/pair and 84% as μg/g adrenal). Daily administration of ACTH to pro-oestrous hypophysectomized females resulted in an increase in adrenal adrenaline content, more pronounced when the results were expressed as μg/g adrenal (87% higher than pro-oestrous hypophysectomy value). The daily treatment of hypophysectomized pregnant rats with ACTH also produced a significant increase in adrenal adrenaline content but in this case, ACTH-induced increases were greater when expressed as μg/pair of glands, a result contrasting with that obtained in non-pregnant females.

The effects of hypophysectomy upon adrenal noradrenaline content in pregnant and pro-oestrous females are illustrated in Figure 6. The operation brought about a slight decrease in non-pregnant rats but similar treatment in pregnancy resulted in a significant decrease in adrenal noradrenaline content, 70% lower when expressed as μg/pair and 59% lower expressed as μg/g adrenal. Daily injections of ACTH for 6 days resulted in increased noradrenaline values in adrenals from both non-pregnant and pregnant females. After ACTH treatment, mean non-pregnant noradrenaline values in hypophysectomized females were not significantly different from those in non-operated rats. Daily ACTH injection to pregnant hypophysectomized females increased adrenal noradrenaline content by 94% (μg/pair of adrenals) or 50% (μg/g adrenal). However, when compared with hypophysectomized non-pregnant rats, noradrenaline content was not restored to control levels.

## Discussion

The results of the present investigation concerning the influence of hypophysectomy upon plasma adrenaline concentration in non-pregnant females are compatible with the suggested role of adrenocortical hormones in the processes of monoamine release and biosynthesis (Axelrod, 1965; 1968). After hypophysectomy of pro-oestrous females, a significant decline in adrenal adrenaline stores results which presumably mirrors the decreased plasma adrenaline content since most circulating adrenaline derives from adrenomedullary secretion (Euler, Franksson & Hellstrom, 1954). Under normal conditions, ACTH release stimulates glucocorticoid production which is essential for the maintenance of adrenaline stores in the adrenal medulla; they cause induction of the medullary enzyme, phenylethanolamine-*N*-methyltransferase (PNMT), responsible for noradrenaline *N*-methylation to adrenaline (Wurtman & Axelrod, 1965, 1966). After hypophysectomy, absence of ACTH secretion prevents adrenal glucocorticoidogenesis which in turn directly affects adrenal adrenaline stores and PNMT activity (Axelrod, 1965; Wurtman, 1966). Hypophysectomy in pregnant females had similar quantitative effects on adrenaline storage and release as in non-pregnant female rats but the reduction in adrenal adrenaline was greater. This finding can be interpreted by taking into consideration the physiological alterations in concentration of corticosteroids and other related hormones during the course of pregnancy. The concentrations of many steroid hormones fluctuate widely during pregnancy, conceivably modifying monoamine storage, release and metabolism in pregnant animals. More specific evidence implicating endocrine gland secretions in monoamine regulation can be seen when hypophysectomized females receive daily injections of ACTH. This treatment increases plasma and adrenal adrenaline concentration to normal levels in pregnant hypophysectomized females; in non-pregnant hypophysectomized females receiving similar treatment the increase was much higher than that observed for pregnant females.

The complete disappearance of plasma adrenaline after adrenalectomy during pro-oestrus and on day 21 post-coitum supports the original assertion of Euler *et al.* (1954) that the adrenal medulla is the main source of adrenaline in the body. Even so, the presence of adrenaline in the plasma of non-pregnant females after adrenalectomy during dioestrus provides evidence in favour of the observations reported by Axelrod (1968) regarding the presence and release of adrenaline in heart and other organs. Some evidence for the presence of extra-adrenal sources of adrenaline is provided by studies on pregnant and non-pregnant adrenalectomized rats receiving hydrocortisone. Such treatment results in the appearance of adrenaline in the plasma. Further support for this hypothesis comes from the fact that there was no evidence of adrenal regeneration in the animals investigated.

The increased plasma noradrenaline content after adrenalectomy in dioestrus and pro-oestrus, as well as on day 21 of pregnancy, points to an activation of adrenergic nerve activity. It has been shown that adrenalectomy increases the activity of central noradrenaline-containing neurones after a certain latency (Javoy, Glowinski & Kordon, 1968). Other observations on the effects of this operation on noradrenaline metabolism and on the role of adrenal hormones in maintaining tissue stores of noradrenaline during increased sympathetic activity (Avakian & Vogt, 1966) are consistent with our findings. Further evidence, relating to stimulation of noradrenergic function due to lack of adrenocortical hormones, is seen in Figure 4 where we record a return of plasma noradrenaline levels to control values after daily administration of hydrocortisone to non-pregnant and pregnant adrenalectomized rats.

Hypophysectomy does not modify adrenal noradrenaline concentration in male rats and this finding is also valid for females hypophysectomized at pro-oestrus. The slight rise observed in plasma noradrenaline concentration in non-pregnant hypophysectomized females may be related to changes in uptake and metabolism (Iversen, 1973). The marked decline in adrenal noradrenaline content in pregnant females after hypophysectomy suggests that noradrenergic activity during pregnancy is more sensitive to changes in pituitary hormone concentrations than that in the non-pregnant state. ACTH treatment restored adrenal noradrenaline values to normal in non-pregnant animals but the mean value in pregnancy remained far below that of controls, thus suggesting the possible involvement of other hormones. The marked increase in plasma noradrenaline concentration after ACTH treatment in both non-pregnant and pregnant hypophysectomized females confirms earlier observations that ACTH has a direct potentiating effect on adrenaline accumulation and secretion (Parvez, Parvez & Roffi, 1974). Our other studies on the role of hypophyseo-adrenocortical function in the processes of monoamine metabolism provide further support for this view (Parvez & Parvez, 1972b; 1974; Holzbauer, Mitchell & Youdim, 1975).

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# SOME OBSERVATIONS ON THE MECHANISM OF BENZODIAZEPINE–BARBITURATE INTERACTIONS IN THE MOUSE

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1 The prolongation of pentobarbitone sleeping times by five benzodiazepines, administered by prior intraperitoneal injection, was measured in mice. The pentobarbitone was injected either intraperitoneally or intracerebroventricularly. For each benzodiazepine, the prolongation was dose-related and differences in potency between benzodiazepines were not marked.

2 The percentage prolongation of sleeping times produced by most of the benzodiazepines was greater when the pentobarbitone was given intracerebroventricularly and was explained by a preferential addition of CNS depressant effects associated with this route.

3 To test whether the action of intraperitoneally administered pentobarbitone had been influenced by a metabolic component, the effects of nitrazepam on drug metabolism, measured by changes in plasma phenazone levels in the mouse, were studied. Nitrazepam (32 mg/kg, i.p.) produced a 23% reduction in the rate of phenazone metabolism.

4 Nitrazepam was also shown to have produced a transient fall in body temperature. Calculations based on $Q_{10}$ values suggested that this hypothermia accounted, at most, for half the metabolic change measured.

## Introduction

The prolongation of barbiturate-induced anaesthesia (sleeping time) by benzodiazepines in laboratory animals has been reported. It has been observed in experiments using different species (Dobkin, 1961; del Pozo & Armas, 1973), different benzodiazepines (Frommel, Fleury, Schmidt-Ginzkey & Béguin, 1960; Hester, Rudzik & Kamdar, 1971) and either pentobarbitone (Jori, Prestini & Pugliatti, 1969), hexobarbitone (Noordhoek, 1968) or thiopentone (Alps, Harry & Southgate, 1973). It has also been reported in man (Tammisto, Elfving, Saikku & Tiitinen, 1967). The prolongation has been assessed in animals either by sleeping time measurements *per se* or by a comparison of the $ED_{50}$ values of barbiturates given with or without benzodiazepine pretreatment (Zbinden, Bagdon, Keith, Phillips & Randall, 1961).

The mechanism of the benzodiazepine-barbiturate interaction has not been fully elucidated. It may involve the addition of central nervous system (CNS) depressant effects; it may involve a metabolic interaction; the rate of penetration of barbiturate into or the rate of its removal from the brain may be altered by benzodiazepine.

In view of the wide variety of experimental variables used in animal studies, and the largely unknown mechanisms of the benzodiazepine-barbiturate interaction, it seemed appropriate to study the effect of a number of clinically used benzodiazepines on pentobarbitone-induced sleeping times in mice under narrowly defined conditions.

The possibility of a metabolic component in the interaction suggested that it be compared both when the pentobarbitone was given intraperitoneally and, in order largely to eliminate hepatic metabolism of pentobarbitone, when administered by intracerebroventricular injection (Stevenson & Turnbull, 1974). To this end, a limited study was also undertaken of the effect of nitrazepam on phenazone metabolism, the latter being a recognized indicator of the activity of hepatic drug metabolizing enzymes (Vessell & Page, 1969) by which barbiturates are also metabolized. In view of the reported hypothermia caused by some benzodiazepines (Gey, 1973) and the possible effect of this hypothermia on drug metabolizing enzymes, changes in body temperature produced by nitrazepam both alone and in combination with phenazone were investigated.

## Methods

Male albino mice, Charles River, CDI strain fed on diet 41B and water *ad libitum* were used throughout. The weight range was 16 to 24 g but for individual experiments the range was not greater than 4 grams. The ambient temperature was maintained throughout at 21 ± 1 °C. All experiments were started at 14 h 00 min except the phenazone metabolism studies which were started at 10 h 00 minutes. Animals were always divided randomly into experimental groups.

### *Pentobarbitone sleeping times by intraperitoneal injection*

Sleeping time was measured as the time between loss of righting reflex and the moment animals regained this reflex. It was determined to the nearest 5 seconds. Each benzodiazepine 0.5, 2, 8 or 32 mg/kg, suspended in 1.5% sodium carboxymethylcellulose (SCMC), or SCMC was injected intraperitoneally 30 min before intraperitoneal pentobarbitone sodium 45 mg/kg in saline. Groups of 10 mice were used for each of these five treatments. Only one benzodiazepine was examined on any given experimental day.

### *Pentobarbitone sleeping times by intracerebroventricular (i.c.v.) injection*

The method used was based on that of Haley & McCormick (1957); 10 µl volumes of pentobarbitone sodium in saline (0.9% w/v NaCl solution) were injected with a 25 µl Hamilton syringe fitted with a 27 gauge needle of 3.5 mm length and having a 17° bevel. The injections were made into the conscious mouse at a point 2 mm rostrally to the lambdoid suture and 1 mm laterally from the saggital suture on the left side. These co-ordinates were judged visually by reference to the positions of the eyes of the mouse and to an imaginary line drawn between the anterior base of the ears and the longitudinal mid-line. The validity of this technique for injection into the lateral ventricles was confirmed post-mortem in brain sections of 10 mice injected with 10% Indian ink in saline. Further validation of the technique was gained retrospectively by examination of the precision of sleeping times in mice subjected to given drug treatments (see Results section) and from the fact that only 7 out of a total of 106 mice given the selected fixed dose of pentobarbitone by intracereboventricular injection failed to lose their righting reflex.

To determine a suitable fixed dose of pentobarbitone for use with the benzodiazepines, the relationship between the dose of pentobarbitone and duration of anaesthesia was investigated. Pentobarbitone sodium expressed as dose/mouse, was injected in a constant volume of 10 µlitre. Benzodiazepines, in the same doses as used in the intraperitoneal experiments, or SCMC were injected intraperitoneally 30 min before pentobarbitone. Again, only one benzodiazepine was examined on any given experimental day.

### *Metabolic studies with phenazone*

A comparison was made of phenazone plasma levels with respect to time in mice pre-treated with nitrazepam or vehicle. The analysis involved a modification of the gas chromatographic method of Prescott, Adjepon-Yamoah & Roberts (1973). Mice, in groups of three, were pretreated with nitrazepam (32 mg/kg i.p.) in SCMC or SCMC followed 30 min later by an injection of phenazone (50 mg/kg i.p.) in saline. At subsequent 6 min intervals from 12 to 54 min a different group of mice was anaesthetized with 2% halothane in a mixture of 33% $O_2$ and 67% $N_2O$ and blood was collected from the retro-orbital plexus. The pooled blood obtained from each group of three mice was extracted in the following manner. To a 0.5 ml sample of pooled plasma was added 0.2 ml 5 mol/l NaOH and 1 ml chloroform containing 12.5 µg phenacetin as internal reference standard in a stoppered glass centrifuge tube which was shaken mechanically for 10 minutes. The aqueous phase and interface were removed by aspiration and added to a further 1 ml of chloroform. Shaking was repeated, the aqueous phase and interface aspirated as before and the residual chloroform added to the first chloroform extract. The combined extract was then carefully decanted into a tapered centrifuge tube which was placed in a water bath at 90 °C for 10 min to remove the chloroform. The residue was dissolved in 20 µl chloroform and 3 µl aliquots were analysed for phenazone. A Perkin-Elmer gas chromatograph model F33 with a 2 m × 1.75 mm o.d. glass column packed with 80–100 mesh Gas-Chrome Q coated with 0.5% SE 30 and 0.5% carbowax 20 M was used. The temperature of the column was 220 °C and that of both the injection port and flame ionization detector was 250 °C. The carrier gas was $N_2$ used at a flow rate of 100 ml/minute.

The whole experiment was repeated on four separate days and the results combined for statistical analysis.

### *Measurement of body temperature*

Oesophageal temperature was measured by the method of Brittain & Spencer (1964), with a thermocouple and Ellab electric thermometer type TE3. Groups of 16 mice were used for each four treatments. At zero time, two of these groups were injected with nitrazepam (32 mg/kg i.p.) in SCMC and the remaining two groups with SCMC. After 30 min, one of the groups of mice pretreated with nitrazepam and one of the groups pretreated with SCMC were injected with phenazone (50 mg/kg i.p.). The remaining two groups were injected with saline. Temperature measurements were made over a period of 3.5 hours.

### *Materials*

The benzodiazepines tested were: chlordiazepoxide (Librium, Roche), diazepam (Valium, Roche), medazepam (Nobrium, Roche), nitrazepam (Mogadon, Roche) and oxazepam (Serenid, Wyeth). Pentobarbitone sodium (Macarthys) and phenazone (BDH Chemicals) solutions in sterile saline (Polyfusor, Boots) were prepared immediately before use. Sodium carboxymethylcellulose (Courlose Gum, P40 grade, British Celanese) solutions were sterilized by autoclaving.

Other materials were halothane (Fluothane, ICI), chloroform (Macfarlan Smith) and phanacetin (Evans Medical).

## Results

### *Pentobarbitone sleeping times by intraperitoneal injection*

The effect of benzodiazepine pretreatment on intraperitoneal pentobarbitone sleeping times is shown in Figure 1. The mean sleeping times in the presence of increasing doses of each benzodiazepine are shown relative to corresponding mean control values in which SCMC was given as pretreatment. None of the mean control sleeping times (range 17.4 to 23.1 min), which had been determined on different experimental days, was shown by *t* tests to be significantly different from each other ($P > 0.05$).

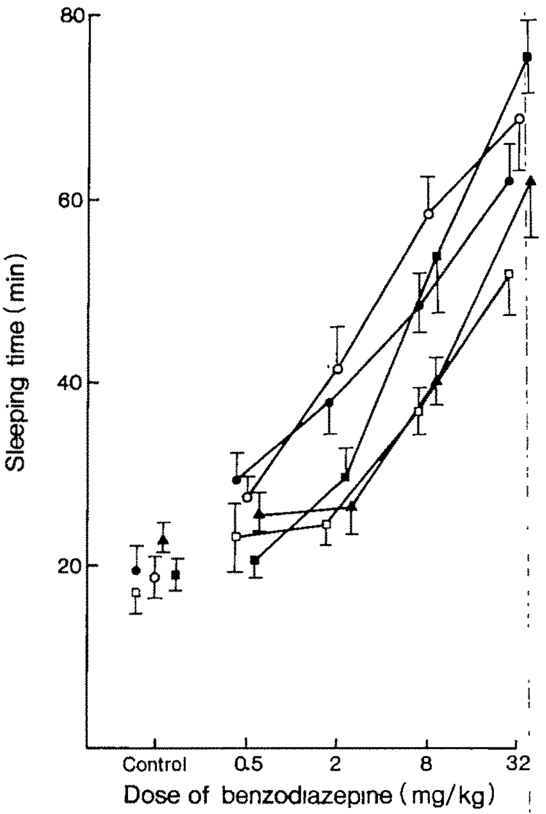

**Figure 1** Effect of intraperitoneal benzodiazepine pretreatment on duration of intraperitoneal pentobarbitone sodium-induced sleeping times in mice. Control animals given sodium carboxymethylcellulose pretreatment. Each point represents the mean of ten observations. Vertical lines represent s.e. mean. (●) Chlordiazepoxide; (■) diazepam; (▲) medazepam; (○) nitrazepam and (□) oxazepam.

A dose-related increase in sleeping time was produced by each benzodiazepine. For both nitrazepam and chlordiazepoxide, a significant prolongation of pentobarbitone sleeping times was produced at all dose-levels ($P < 0.05$). Diazepam and oxazepam showed significant prolongation at doses of 2, 8 and 32 mg/kg whilst for medazepam prolongation was only significant at 8 and 32 mg/kg.

At the 32 mg/kg dose level, it was shown that only the difference between the prolongation with diazepam and oxazepam was significant.

### *Pentobarbitone sleeping times by intracerebroventricular injection*

Figure 2 shows the relationship between dose of pentobarbitone sodium and duration of anaesthesia. Above 200 μg/mouse fatalities occurred; at 250 μg/mouse 4 out of 10 mice died due to respiratory arrest. Below 100 μg/mouse loss of righting reflex was not consistently attained. Thus 125 μg/mouse was chosen for the subsequent experiments in order to produce anaesthesia of a consistently short duration.

The effect of intraperitoneal benzodiazepine pretreatment on intracerebroventricular pentobarbitone sleeping times is shown in Figure 3. Again, none of the mean control sleeping times (range 2.4–3.1 min) were significantly different from each other ($P > 0.05$).

Nitrazepam produced a significant prolongation of sleeping time at all dose levels, diazepam at 2, 8 and 32 mg/kg, medazepam at 8 and 32 mg/kg, whilst for chlordiazepoxide and oxazepam the prolongation was only significant at 32 mg/kg.

At the highest dose level the effect produced by diazepam was significantly greater than that of the other benzodiazepines. It was noted at this dose of benzodiazepine that when the sleeping times were ranked numerically from the most potent, the same order was found as in the intraperitoneal sleeping time experiments: diazepam; nitrazepam; medazepam; chlordiazepoxide; oxazepam.

The dose-related prolongation of the sleeping time confirms and extends the results previously reported in the rat (Stevenson & Turnbull, 1974).

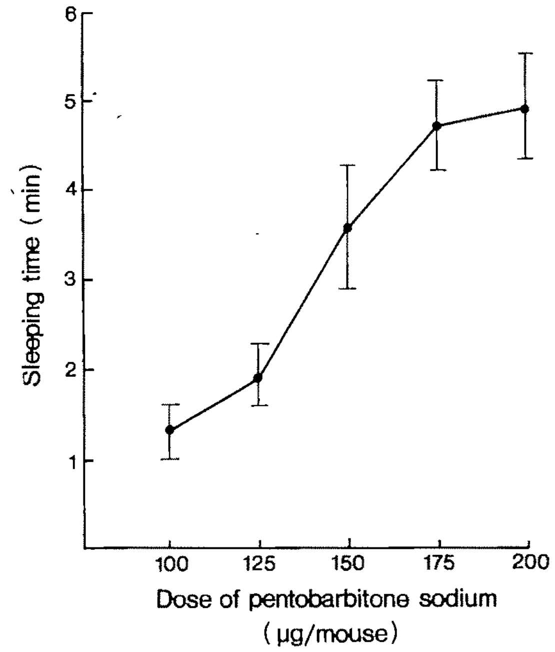

**Figure 2** The relationship between dose of intracerebroventricularly administered pentobarbitone sodium and duration of anaesthesia. Each point represents the mean of at least 12 observations. Vertical lines represent s.e. mean.

### *Metabolic studies with phenazone*

Figure 4 shows the decline in plasma levels of phenazone in mice pretreated (i.p.) with either nitrazepam (32 mg/kg) or SCMC. Each point is the mean of 4 determinations, the 4 determinations being made of different days. The lines were fitted by regression analysis, the errors of the slopes being calculated from the value of $Sb/_b$ where Sb is the standard error of the regression coefficient (b) of the plot. These errors are 4% and 7.5% after nitrazepam and SCMC pretreatment, respectively. A test for parallelism (Diem & Lentner, 1970) indicated that the regression lines were not parallel.

The half life $T_{\frac{1}{2}}$ of phenazone in the presence of SCMC or nitrazepam was 22.6 and 29.2 min respectively, the corresponding elimination rate constants ($K_e$) being $3.07 \times 10^{-2}$ and $2.37 \times 10^{-2}$/minute. The constants were calculated from $K_e = 0.693/T_{\frac{1}{2}}$ (Riggs, 1963). Thus nitrazepam caused a 23% decrease in the rate of phenazone metabolism.

### *Body temperature after intraperitoneal injections of nitrazepam and phenazone*

The body temperature of both groups of mice injected with 32 mg/kg nitrazepam reached its lowest value at 50 min (Figure 5). These falls were short-lived. The maximum fall was measured in those mice receiving a combination of nitrazepam and phenazone and was only 1°C. A comparison of the temperature fall at 50 min in groups of mice receiving nitrazepam with the temperature of the corresponding control groups, showed that only in the case of combined nitrazepam and phenazone treatment, was the fall significant ($P < 0.05$). The body temperature of mice receiving phenazone without nitrazepam showed no change.

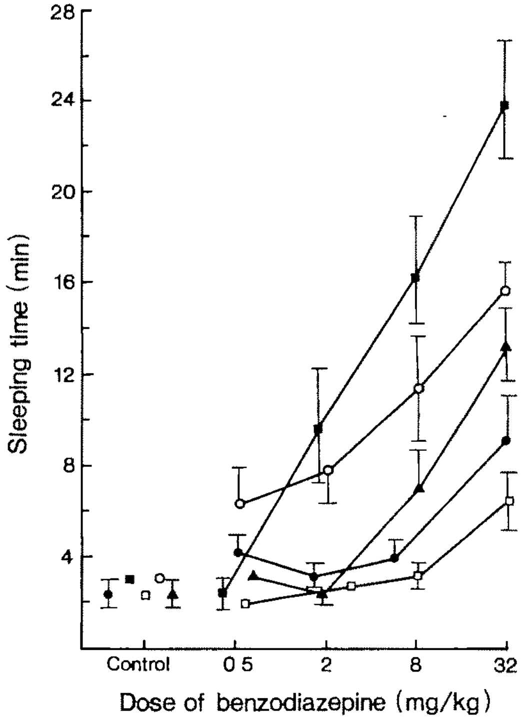

**Figure 3** Effect of intraperitoneal benzodiazepine pretreatment on duration of intracerebroventricularly administered pentobarbitone sodium-induced sleeping times in mice. Control animals given sodium carboxymethylcellulose pretreatment. Each point represents the mean of ten observations. Vertical lines represent s.e. mean. (●) Chlordiazepoxide; (■) diazepam; (▲) medazepam; (○) nitrazepam and (□) oxazepam.

## Discussion

While the fact of an interaction between barbiturates and benzodiazepines is indisputable, the mechanism is little understood. The present study confirms the ability of the benzodiazepines to prolong intraperitoneal pentobarbitone sleeping time in the mouse.

This interaction has been explained in terms of an addition of barbiturate-induced CNS depression to

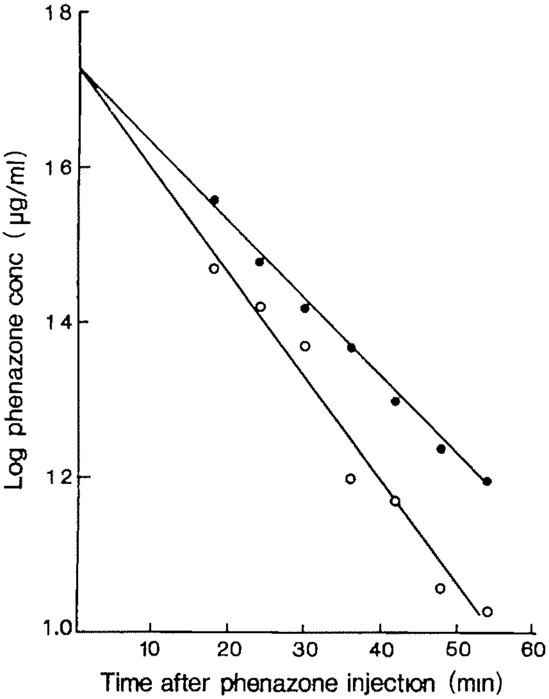

**Figure 4** The effect of nitrazepam on phenazone plasma concentration. Nitrazepam 32 mg/kg (●) or sodium carboxymethylcellulose (○) injected intraperitoneally 30 min before phenazone (50 mg/kg, i.p.). Each point is the mean of 4 determinations; lines calculated by regression analysis.

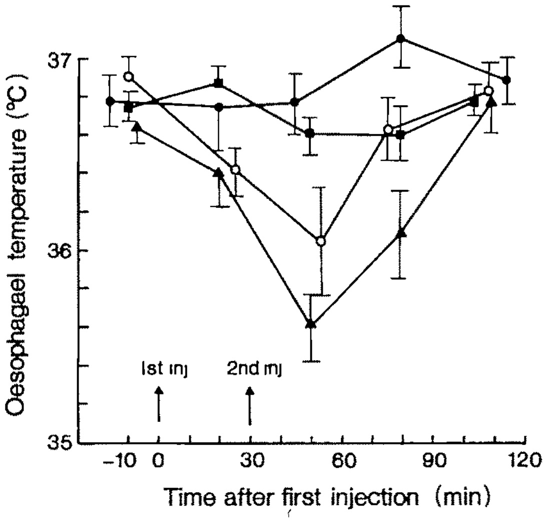

**Figure 5** Differences in oesophageal temperature in mice injected with (●) sodium carboxymethylcellulose (SCMC) followed by saline; (■) SCMC followed by phenazone 50 mg/kg; (▲) nitrazepam, 32 mg/kg, followed by phenazone, 50 mg/kg, and (○) nitrazepam, 32 mg/kg, followed by saline. In each treatment the first injection was 30 min before the second. Each point represents the mean of 16 determinations. Vertical lines represent s.e. mean.

the more selective depression produced by benzodiazepines (Stevenson and Turnbull, 1974). It has also been suggested that the interaction may involve potentiation rather than simple addition (Dobkin, 1961; Heckmatt, Houston, Clow, Stephenson, Dodd, Lealman & Logan, 1976). The only study to measure potentiation however was that of Alps *et al.* (1973) who employed the isobologram plot (Loewe, 1957) in an investigation into the interaction of diazepam and lorazepam with thiopentone.

Pentobarbitone is inactivated almost exclusively by liver microsomal enzymes (Cooper & Brodie, 1957). Drugs, classically SKF 525A, which inhibit these enzymes can prolong barbiturate-induced anaesthesia without themselves causing anaesthesia (Cook, Macko & Fellows, 1954). In the present study, benzodiazepines, even at low doses, doubled the pentobarbitone sleeping time without themselves causing loss of righting reflex. Thus there could be a metabolic component contributing to this interaction. The intracerebroventricular administration of pentobarbitone after benzodiazepine was an attempt to eliminate any metabolic interactions because pentobarbitone is not metabolized in mouse brain (Sauberman, Gallagher & Hedley-Whyte, 1974). The benzodiazepine prolongation in mice was dose-related, diazepam being the most potent compound tested. If, in general, the mechanism of the benzodiazepine-barbiturate interaction includes a significant metabolic component then the prolongation of intraperitoneal pentobarbitone sleeping time should be greater than when pentobarbitone was given by the intracerebroventricular route. However, when those results are considered and compared with their respective controls it is clear that there is a greater percentage prolongation following intracerebroventricular injection than following intraperitoneal injection. These data are not inconsistent with a metabolic interaction between the benzodiazepines and intraperitoneal pentobarbitone. However, the intracerebroventricular results require a different explanation.

Injection of the chosen volume of pentobarbitone solution into cerebral ventricles by the technique of Haley & McCormick (1957) causes the majority of the injection to flood the ventricular system, although some solution is immediately lost to the periphery along the needle tract. Additional loss of solution into the saggital sinus may occur through arachnoid villi ruptured by the technique (Shaw, 1974). As pentobarbitone is very lipid soluble at physiological pH, diffusion will rapidly occur from ventricular system into neuronal tissue along its concentration gradient. This was shown in rats (Stevenson & Turnbull, 1970) with $^{14}$C-labelled pentobarbitone, the subsequent fall in drug concentration from neuronal tissue by diffusion and removal into blood producing an anaesthesia of short duration. A detailed study of [$^{14}$C]-pentobarbitone injected intravenously in mice has shown an uneven brain distribution of this compound (Saubermann *et al.*, 1974). This distribution changes not only absolutely but also relatively with time and is related to blood flow. It might be expected that brain distribution of pentobarbitone would be different when given by the two routes used in the present study. It is possible, therefore, that a central additive effect may exist between pentobarbitone given by intrecerebroventricular injection and benzodiazepine in a brain area which receives a lower pentobarbitone concentration by the intraperitoneal route. This could explain the greater effect of benzodiazepines on centrally administered pentobarbitone.

Barbiturates are metabolized by liver microsomal enzymes (Cooper & Brodie, 1957); phenazone half-lives are a good measure of the activity of these enzymes (Vessell & Page, 1969). Thus an investigation of the effect of nitrazepam, one of the most potent of the benzodiazepines tested in the present work, on the half-life of phenazone was carried out. The results show a 23% decrease in the rate of phenazone metabolism and a concomitant increase in half-life in mice treated with nitrazepam.

Prazepam a newly-developed long-acting benzodiazepine, has been shown to cause an increase in phenazone half-lives in man (Vesell, Passananti, Viau, Epps & Di Carlo, 1972). Although the present study has shown the effect of acute nitrazepam pretreatment on phenazone metabolism, it should be recognized that the effect of benzodiazepines may be different on chronic administration. There is evidence that after chronic administration some benzodiazepines may cause induction of drug metabolizing enzymes (Hoogland, Miya & Bousquet, 1966; Vorne & Idänpään-Heikkilä, 1975).

The present work showed a maximum fall in body temperature of 1°C after nitrazepam and the corresponding change in the rate of metabolism attributable to this hypothermia was calculated from $Q_{10}$ values (Heintzen, 1958). For most drugs, $Q_{10}$ values are between 2 and 2.5 (Prosser, 1973); assuming a value of 2.5, the change in the rate of metabolism is 9.6% for the 1°C fall in body temperature. This value represents the maximum metabolic change attributable to the hypothermia as the temperature fall was not sustained. These findings suggest that, at most, half the change in phenazone metabolism brought about by nitrazepam may be due to induced hypothermia and the remainder is probably due to metabolic interaction.

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# DRUGS ALTERING INSULIN SECRETION: EFFECTS ON PLASMA AND BRAIN CONCENTRATIONS OF AROMATIC AMINO ACIDS AND ON BRAIN 5-HYDROXYTRYPTAMINE TURNOVER

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1 An investigation was made into the effects of drugs which alter insulin secretion on the concentrations of tryptophan and other aromatic amino acids in plasma and brain and on 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in brain. Drugs used were streptozotocin, propranolol, tolbutamide and phentolamine.

2 Tolbutamide and phentolamine increased the plasma insulin concentrations by 100% and 300% respectively but with little effect on the brain/plasma ratios for the aromatic amino acids. Previously propranolol was found to decrease plasma insulin by 50% without altering the above ratios. The ratios were decreased by streptozotocin but only when plasma insulin fell by more than 50%.

3 Phentolamine and propranolol did not alter the brain/plasma ratios for the aromatic amino acids in streptozotocin-treated rats.

4 The results suggest that only large changes of insulin secretion e.g. those associated with food intake or aminophylline injection are likely to alter appreciably the brain/plasma ratios for the aromatic amino acids.

5 Tolbutamide displaced tryptophan from its binding to plasma albumin and increased brain 5-HIAA probably by inhibiting 5-HIAA efflux from brain. The other drugs did not alter brain 5-HT or 5-HIAA concentrations.

## Introduction

When brain tryptophan concentration changes it can affect 5-hydroxytryptamine (5-HT) synthesis because tryptophan hydroxylase, the rate limiting enzyme for 5-HT synthesis is normally unsaturated with its substrate tryptophan (Eccleston, Ashcroft & Crawford, 1965; Friedman, Kappelman & Kaufman, 1972). Furthermore, recent work shows that quite moderate changes of brain tryptophan concentration can lead to altered behaviour (Lytle, Messing, Fisher & Phebus, 1975; Taylor, 1976; Bloxam, Curzon, Kantamaneni & Tricklebank, 1977). Therefore mechanisms by which brain tryptophan concentration is altered are of some importance. One determinant of brain tryptophan concentration is insulin secretion as this can result in decreased plasma concentrations of large neutral amino acids which compete with tryptophan for uptake by brain (Fernstrom & Wurtman, 1971; 1972; Madras, Cohen, Messing, Munro & Wurtman, 1974). Experiments with insulin and two drugs, aminophylline and streptozotocin, which alter its secretion suggest that the above mechanism and altered binding of plasma tryptophan to albumin can concurrently affect brain tryptophan concentration (Curzon & Fernando, 1976; Fernando, Knott & Curzon, 1976). Also the increase of plasma insulin following injection of the 5-HT receptor antagonist, methiothepin, has been suggested as a partial explanation of the increased brain 5-HT synthesis caused by this drug. (Jacoby, Shabshelowitz, Fernstrom & Wurtman, 1975).

It was of interest therefore to investigate whether drugs known to alter insulin secretion also alter the concentrations of tryptophan and other aromatic amino acids in plasma, brain and muscle and the concentrations of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in brain. Drugs used were streptozotocin and propranolol which decrease insulin secretion and tolbutamide and phentolamine which increase it.

## Methods

Male Sprague-Dawley rats (ALA, Alconbury, Huntingdon, U.K.), 180–220 g were kept 3 or 4 to a cage in an acoustically lagged chamber at 24 ± 3°C on a 18 h 00 min–06 h 00 min light-dark cycle and fed with Oxoid 41B pellets and water *ad libitum*.

The following drugs were used: phentolamine (Ciba Ltd.) dissolved in 0.9% w/v NaCl solution (saline); probenecid (Sigma Ltd.) dissolved in the minimum volume required of 1N NaOH, diluted with saline and pH adjusted to 9; (±)-propranolol (ICI Ltd.) dissolved in saline; streptozotocin (Upjohn, Kalamazoo, USA) dissolved in 0.07M sodium citrate buffer (pH 4.5) immediately before use, tolbutamide ('Rastinon' injection, Hoechst Ltd.). Drugs were injected intraperitoneally, 2.5 ml/kg body wt except for tolbutamide which was injected intraperitoneally, 5.0 ml/kg body wt. Control animals were injected with the appropriate vehicles.

At various intervals after injection as indicated in the Results section rats were killed by guillotine (between 15 h 00 min and 16 h 00 min) and the blood drained into tubes containing citrate. The plasma was separated by centrifugation and stored at −20°C. Brain and gastrocnemius muscle were removed, frozen immediately on solid $CO_2$ and stored at −20°C. Plasma unesterified fatty acids (UFA), insulin, tryptophan (total and free), tyrosine, phenylalanine, 5-HT and 5-HIAA and muscle tryptophan, tyrosine and phenylalanine were determined by methods described previously (Curzon & Fernando, 1976). The plasma was ultrafiltered for separation of free tryptophan at non-physiological pH (7.8–7.9) as pH rose during storage. Although the resultant *in vitro* decrease of free tryptophan concentration (McMenamy & Seder, 1963) would have apparently increased the ratios of tissue tryptophan to plasma free tryptophan concentrations, this effect would have been comparable in all experiments as plasma pH rose on storage independently of the drug treatment used.

## Results

### *Effects on aromatic amino acid disposition of drugs decreasing plasma insulin concentration*

*Streptozotocin.* Streptozotocin destroys pancreatic insulin secreting cells (Arison, Ciaccio, Glitzer, Cassaro & Pruss, 1967) and thus decreases plasma insulin concentration. Table 1 shows the results of two separate experiments in which streptozotocin was injected 72 h before rats were killed. Intravenous injection of 65 mg/kg of the drug led to a considerable fall of plasma insulin in association with a large and significant rise of free tryptophan. Despite this increased availability of tryptophan its concentration in the brain fell significantly and the ratio of brain tryptophan to plasma free tryptophan showed a large significant fall (−65%). These results were similar to previous findings (Fernando *et al.*, 1976) except for the fall of brain tryptophan concentration. It may be that this difference was due to the lower plasma insulin levels attained in the previous experiment.

Intraperitoneal injection appeared less effective as 90 mg/kg (i.p.) of streptozotocin led to a smaller (though significant) fall in plasma insulin. This was associated with small and non-significant changes in the brain tryptophan/plasma free tryptophan ratio (−20%) and in the corresponding ratios for the other aromatic amino acids (not shown).

*Propranolol.* Insulin secretion is increased by the action of catecholamines at pancreatic adrenoceptors (Turtle, Littleton & Kipnis, 1967). Previously the β-adrenoceptor blocking agent, propranolol was found to decrease plasma insulin significantly (Curzon & Fernando, 1976). The fall of insulin was similar to that found after streptozotocin injection (90 mg/kg i.p.) and brain tryptophan and its ratio to plasma free tryptophan were similarly unaffected. As it was conceivable that propranolol might have an action on tryptophan distribution which was not mediated by insulin and which might obscure the effects of insulin changes, propranolol was given to streptozotocin-treated rats (Table 2). Plasma insulin and both plasma and brain tryptophan concentrations were not significantly altered. Plasma and brain tyrosine or phenylalanine concentrations were also unaffected (results not shown).

### *Effects on aromatic amino acid disposition of drugs increasing plasma insulin concentration*

*Tolbutamide.* Sulphonylureas such as tolbutamide increase pancreatic insulin secretion probably by increasing islet cell cyclic adenosine 3′,5′-monophosphate (Goldfine, Perlman & Roth, 1971). Table 3 shows the results of separate experiments in which rats were given tolbutamide and killed 1 h or 3 h later. Plasma insulin was considerably lower in the control group killed 1 h after injection of saline than in the corresponding control group killed 3 h after injection. The lower values in the former group may reflect a transient injection stress-provoked decrease of insulin secretion (Porte & Robertson, 1973). Plasma free tryptophan concentrations were unaltered but as total tryptophan fell significantly the percentage of free tryptophan rose. These tryptophan changes were most pronounced in rats killed 1 h after tolbutamide injection and occured without a significant UFA increase. The following observation suggests that they were probably due to the freeing by tolbutamide of tryptophan bound to plasma protein i.e. the addition of 4.5 mg tolbutamide dissolved in 0.2 ml saline to 1.0 ml plasma resulted in 81% of the plasma tryptophan becoming ultrafilterable. (The addition of 0.2 ml saline to 1.0 ml plasma resulted in only 11% of the tryptophan being ultrafilterable). The effect of tolbutamide was not due to the alteration of plasma pH as this was unchanged.

Brain tryptophan and its ratio to plasma free tryptophan increased slightly but except for the increase of brain tryptophan concentration 1 h after tolbutamide injection (Figure 1) the changes were not significant. Plasma values for tyrosine and phenylalanine (Table 4) were essentially unaltered while the

**Table 1** Effect of streptozotocin on plasma concentrations of unesterified fatty acid (UFA), insulin and tryptophan and on brain concentrations of tryptophan

| | *Saline (8)* | *Streptozotocin (90 mg/kg i.p.) (9)* | *Saline (4)* | *Streptozotocin (65 mg/kg i.v.) (4)* |
|---|---|---|---|---|
| Plasma UFA (mEq/l) | 0.71±0.40 | 0.79±0.18 | 0.44±0.16 | 0.83±0.32 |
| Plasma insulin (μu/ml) | 28.4±6.5 | 14.7±6.4**** | 31.8±11.6 | 6.8±3.9** |
| *Tryptophan* | | | | |
| Plasma total (μg/ml) | 18.94±2.54 | 18.15±2.08 | 17.92±1.09 | 13.51±6.82 |
| Plasma free (μg/ml) | 1.94±0.43 | 2.44±0.39* | 1 82±0.29 | 3.59±0.84*** |
| Brain (μg/g wet wt) | 4.94±0.70 | 5.09±0.49 | 4.20±0.26 | 2.78±0.62*** |
| Brain/plasma free | 2.67±0.74 | 2.15±0.46 | 2.36±0.42 | 0.83±0.33*** |

Rats were killed 72 h after injection of streptozotocin. Controls were injected with saline. Results are expressed as means±one s.d. Nos of determinations are shown in parentheses. Results compared by Student's *t* test.
Differences from controls: *$P<0.05$; **$P<0.02$; ***$P<0.01$; ****$P<0.001$

**Table 2** Effect of propranolol on plasma concentrations of unesterified fatty acid (UFA), insulin and tryptophan and on brain concentrations of tryptophan in streptozotocin treated rats

| | *Saline (7–8)* | *Propranolol (7)* |
|---|---|---|
| Plasma UFA (mEq/l) | 0.80±0.30 | 0.87±0.14 |
| Plasma insulin (μu/ml) | 12.4±7.9 | 15 0±7.7 |
| *Tryptophan* | | |
| Plasma total (μg/ml) | 13.18±2.65 | 11.54±1.99 |
| Plasma free (μg/ml) | 1.39±0.22 | 1 44±0 37 |
| Brain (ug/g wet wt) | 3.15±0.41 | 2.75±0.45 |
| Brain/plasma free | 2.27±0.19 | 2.09±0.26 |

Propranolol (2 mg/kg i.p. x 2) was injected 3 h and 1 h before killing. Streptozotocin (90 mg/kg i.p.) was given 48 h earlier. Controls were injected with saline instead of propranolol. Results are expressed as means±one s.d. Nos of determinations are shown in parentheses. Results compared by Student's *t* test.
No significant differences.

**Table 3** Effect of tolbutamide on plasma concentrations of unesterified fatty acid (UFA), insulin and tryptophan and on brain concentrations of tryptophan

| | *Experiment 1* | | *Experiment 2* | |
|---|---|---|---|---|
| | *Saline (7–8)* | *Tolbutamide (7–8)* | *Saline (5–6)* | *Tolbutamide (5–6)* |
| | *Killed at 3 h* | | *Killed at 1 h* | |
| Plasma UFA (mEq/l) | 0.31±0.18 | 0.38±0.15 | 0.71±0.30 | 0.51±0.22 |
| Plasma insulin (μu/ml) | 25.0±8.0 | 41.0±19.0* | 9.5±3.0 | 18.2±8.7* |
| *Tryptophan* | | | | |
| Plasma total (μg/ml) | 16.29±2.63 | 11.33±4.06** | 16.46±3.61 | 5.73±0.54*** |
| Plasma free (μg/ml) | 1.32±0.35 | 1.29±0.22 | 1.21±0.27 | 1.25±0.17 |
| Brain (μg/g wet wt) | 5.01±0.59 | 5.51±0.46 | 4.41±0.39 | 4.95±0.24 |
| Brain/plasma free | 4.01±1.01 | 4.35±0.56 | 3.77±0.70 | 4.02±0.49 |

Rats were killed either 1 or 3 h after injection of tolbutamide (230 mg/kg i.p.) as the Na salt. Controls were injected with saline. Results are expressed as means±one s.d. Nos of determinations are shown in parentheses. Results compared by Student's *t* test.
Differences from controls: *$P<0.05$; **$P<0.02$; ***$P<0.001$

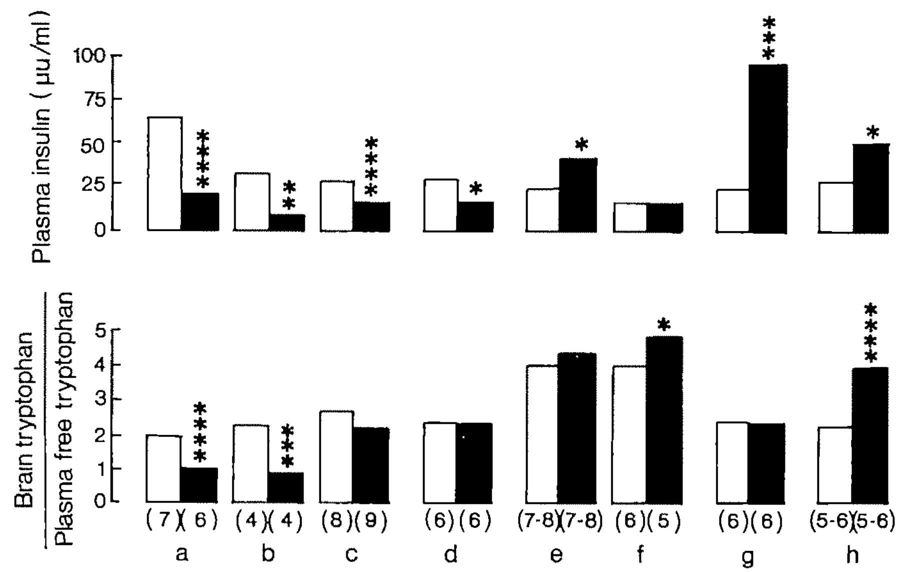

**Figure 1** Effects of drugs on plasma insulin concentration and on the ratios of brain tryptophan to plasma free tryptophan concentrations. (a) Streptozotocin, 65 mg/kg i.v. (72 h); (b) streptozotocin, 65 mg/kg i.v. (72 h); (c) streptozotocin, 90 mg/kg i.p. (72 h); (d) propranolol, 2 mg/kg i.p. x 2 (1 h, 3 h); (e) tolbutamide, 230 mg/kg i.p. (3 h); (f) tolbutamide, 230 mg/kg i.p. (3 h) plus probenecid, 200 mg/kg i.p. x 2 (1 h, 3 h); (g) phentolamine, 20 mg/kg i.p. x 2 (1 h, 3 h); (h) aminophylline 150 mg/kg i.p. (3 h). Drugs were injected at times before the animals were killed as shown in parentheses. Results in (a) from Fernando *et al.* (1976). Significance of differences from controls: $^*P<0.05$; $^{**}P<0.02$; $^{***}P<0.01$; $^{****}P<0.001$

brain showed a slight increase so that brain/plasma ratios also increased slightly. The latter increase was significant for tyrosine only.

*Phentolamine.* Drugs blocking $\alpha$-adrenoceptors increase plasma insulin (Turtle *et al.*, 1967). Thus phentolamine caused a very considerable increase of plasma insulin (Table 5). Changes of plasma UFA, plasma total and free tryptophan and brain tryptophan were negligible so that the ratio of the latter to plasma free tryptophan was not significantly altered. Muscle tryptophan concentration was also unaffected. When phentolamine was given to rats treated with streptozotocin in order to reveal any

**Table 4** Effect of tolbutamide on plasma and brain concentrations of tyrosine and phenylalanine

| | *Saline (6)* | Tolbutamide (5) |
|---|---|---|
| | *Killed at 1 h* | |
| *Tyrosine* | | |
| Plasma (μg/ml) | 11.45±1.28 | 10.76±1.02 |
| Brain (μg/g wet wt) | 9.04±0.52 | 10.32±1.00* |
| Brain/plasma | 0.80±0.09 | 0.97±0.14* |
| *Phenylalanine* | | |
| Plasma (μg/ml) | 7.66±1.08 | 7.29±0.70 |
| Brain (μg/g wet wt) | 12.90±0.56 | 14 55±1.48* |
| Brain/plasma | 1.72±0.24 | 2 02±0.35 |

Rats were killed either 1 or 3 h after injection of tolbutamide (230 mg/kg i.p.) as the Na salt Controls were injected with saline. Results are expressed as means±one s.d. Nos of determinations are shown in parentheses. Results compared by Student's *t* test.
Differences from controls: $^*P<0.05$.

**Table 5** Effect of phentolamine on plasma concentrations of unesterified fatty acid (UFA) and insulin and on the disposition of tryptophan

| | | | *Streptozotocin treated* | |
|---|---|---|---|---|
| | *Saline (6)* | *Phentolamine (6)* | *Saline (7–8)* | *Phentolamine (7–8)* |
| Plasma UFA (mEq/l) | 0.57±0.18 | 0.65±0.36 | 0.80±0.30 | 1.12±0.13* |
| Plasma insulin (μu/ml) | 24.2±10 2 | 95.5±45** | 11.3±5.70 | 15.0±7.70 |
| *Tryptophan* | | | | |
| Plasma total (μg/ml) | 12.19±2.30 | 11.13±2.69 | 13.18±2.65 | 8.56±1.42*** |
| Plasma free(μg/ml) | 1 34±0.34 | 1.62±0.44 | 1 39±0.22 | 1.99±0.87 |
| Muscle (μg/g wet wt) | 4.17±0.76 | 3.96±0.36 | 3.75±0.79 | 3.85±1.00 |
| Brain (μg/g wet wt) | 3.45±0.45 | 3.42±0.53 | 3.15±0.41 | 3.35±0.34 |
| Brain/plasma free | 2.39±0.37 | 2.29±0.67 | 2.27±0.19 | 1 94±0.75 |

Phentolamine (20 mg/kg i.p. x 2) was injected 3 h and 1 h before killing. Streptozotocin (90 mg/kg I p ) was given 48 h earlier. Controls were injected with saline. Results expressed as means±one s.d. Nos of determinations are shown in parentheses. Results compared by Student's *t* test.
Differences from controls: **P*<0.02; ***P*<0.01; ****P*<0.001

effects on amino acid distribution not mediated by insulin then plasma insulin concentrations were no longer significantly altered (Table 5), plasma UFA increased and total tryptophan fell significantly. Muscle tryptophan concentration and the ratio of brain tryptophan to plasma free tryptophan (as in the absence of streptozotocin) were not significantly altered.

Table 6 shows tyrosine and phenylalanine concentrations in the above groups of animals. Phentolamine injection led to significant decreases of tyrosine in plasma, muscle and brain. The ratio of brain to plasma concentrations was slightly increased. Comparable tyrosine changes occurred when phentolamine was given to streptozotocin-treated rats. Phentolamine did not significantly alter tissue phenylalanine concentrations in normal rats but caused small but significant decreases in both muscle and brain after streptozotocin treatment. The ratio of brain to plasma concentrations was not significantly changed.

### *Effects on 5-hydroxytryptamine metabolism of drugs altering plasma insulin concentration*

Table 7 summarizes changes of brain tryptophan, 5-HT and 5-HIAA concentrations in the above experiments. Streptozotocin, propranolol or phentolamine had negligible effects on 5-HT or 5-HIAA. This was found even after 65 mg/kg streptozotocin intravenously which significantly decreased brain tryptophan concentrations.

**Table 6** Effect of phentolamine on the disposition of tyrosine and phenylalanine

| | | | *Streptozotocin treated* | |
|---|---|---|---|---|
| | *Saline (6)* | *Phentolamine (6)* | *Saline (7–8)* | *Phentolamine (7–8)* |
| *Tyrosine* | | | | |
| Plasma (μg/ml) | 13.87±2.23 | 9.08±1.57*** | 15.90±2.29 | 10.76±1.50**** |
| Muscle (μg/g wet wt) | 15.17±1.51 | 11.50±2.01*** | 12.47±1.57 | 8.87±1.81**** |
| Brain (μg/g wet wt) | 9.74±0.62 | 7.90±1.19*** | 12.64±1.90 | 9.63±1.07*** |
| Brain/plasma | 0.71±0.08 | 0.88±0.12** | 0.77±0.05 | 0.94±0.23 |
| *Phenylalanine* | | | | |
| Plasma (μg/ml) | 9.29±0.95 | 9.36±1.00 | 9.53±2.28 | 8 97±1.38 |
| Muscle (μg/g wet wt) | 5.75±0.59 | 6.54±1.20 | 12.47±1.57 | 8.87±1.81**** |
| Brain (μg/g wet wt) | 9.80±0.79 | 10.88±1.68 | 11.91±1.30 | 10.67±0.78* |
| Brain/plasma | 1.06±0.12 | 1.19±0.30 | 1.32±0.35 | 1.19±0.12 |

Phentolamine (20 mg/kg i.p. x 2) was injected 3 h and 1 h before killing. Streptozotocin (90 mg/kg i p.) was given 48 h earlier. Controls were injected with saline. Results expressed as means±one s.d. Nos of determinations are shown in parentheses. Results compared by Student's *t* test.
Differences from controls: **P*<0.05; ***P*<0.02; ****P*<0.01; *****P*<0.001

**Table 7** Effect of drugs on brain 5-hydroxytryptamine (5-HT) metabolism

| | *Brain concentrations (µg/g wet wt)* | | |
|---|---|---|---|
| *Drugs administered* | *Tryptophan* | *5-HT* | *5-HIAA* |
| Saline (8) | 4.94±0.70 | 0.63±0.24 | 0.48±0.09 |
| Streptozotocin 90 mg/kg i.p. 72 h (9) | 5.09±0.49 | 0.72±0.01 | 0.55±0.06 |
| Saline (4) | 4.20±0.26 | 0.61±0.01 | 0.56±0.08 |
| Streptozotocin 65 mg/kg i.v. 72 h (4) | 2.78±0.62* | 0.66±0.07 | 0.56±0.05 |
| Saline (6)[1] | 2.47±0.27 | 0.52±0.05 | 0.45±0.08 |
| Propranolol 2 mg/kg i.p. × 2; 1 h, 3 h (6)[1] | 2.75±0.33 | 0.56±0.04 | 0.49±0.03 |
| Saline (8) | 5.01±0.59 | 0.62±0.08 | 0.48±0.04 |
| Tolbutamide 230 mg/kg i.p.3 h (7) | 5.51±0.46 | 0.68±0.04 | 0.80±0.07** |
| Probenecid 200 mg/kg i.p. × 2; 1 h, 3 h (6) | 4.71±0.57 | 0.87±0.07 | 0.93±0.10 |
| Probenecid 200 mg/kg i.p. × 2; 1 h, 3h<br>Tolbutamide 230 mg/kg i.p. 3 h (5) | 8.78±1.09** | 0.95±0.08 | 1.20±0.08** |
| Saline (6) | 3.45±0.45 | 0.54±0.04 | 0.49±0.05 |
| Phentolamine 20 mg/kg i.p. × 2; 1 h, 3 h (6) | 3.42±0.53 | 0.57±0.03 | 0.54±0.07 |

Results expressed as means ± one s.d. Nos of determinations are shown in parentheses. Results compared by Student's *t* test.
Differences from controls: *$P<0.01$; **$P<0.001$
[1] Results from Curzon & Fernando (1976).

Tolbutamide caused a considerable and highly significant increase of brain 5-HIAA (+67%) 3 h later which was proportionately much greater than the increases of tryptophan (+12% at 1 h, +10% at 3 hours). When rats were also given probenecid to inhibit efflux of 5-HIAA from the brain, tolbutamide caused a relatively small increase of 5-HIAA (+29%) associated with a much larger increase of tryptophan (+86%).

## Discussion

The results of drug treatments described here or elsewhere (Curzon & Fernando, 1976; Fernando *et al.*, 1976) showed that large changes of plasma insulin concentration can alter the ratios of the concentrations of aromatic amino acids in brain to their concentrations in plasma. The results are summarized in Figure 1.

Thus after streptozotocin injection (65 mg/kg i.v.) the plasma concentrations of insulin, the ratio for the concentrations of plasma free tryptophan to brain tryptophan (Figure 1a, b) and the corresponding ratios for tyrosine and phenylalanine (Fernando *et al.*, 1976) showed large falls. Conversely aminophylline increased these parameters (Curzon & Fernando, 1976 and Figure 1h). Also the absolute concentrations of tryptophan in the brain were decreased by streptozotocin (Table 1) and increased by aminophylline. The changes of brain concentration of tryptophan and of the other aromatic amino acids were less striking than the changes in the plasma-brain ratios (Fernando *et al.*, 1976) (Table 1); in some circumstances they were absent (Curzon & Fernando, 1976; Fernando *et al.*, 1976).

Other experiments with drugs indicated that although the above large changes of plasma insulin concentration were associated with major changes in the disposition of the aromatic amino acids between plasma and brain, quite appreciable but smaller insulin changes were not associated with alterations of amino acid disposition e.g. propranolol or streptozotocin (90 mg/kg i.p.) led to significant and proportionately comparable falls of plasma insulin but without significant or considerable changes of tryptophan disposition (Figure 1c, d). Furthermore, while tolbutamide did significantly increase both plasma insulin (Figure 1e) and aromatic amino acid concentrations in the brain (Tables 3, 4) the latter changes were relatively small and only in the case of tyrosine was the ratio of the brain to plasma concentrations significantly increased. The ratio of brain tryptophan to plasma free tryptophan concentration increased slightly but significantly when tolbutamide was given to probenecid-treated rats although interpretation of this result is difficult as plasma insulin concentration was not raised.

Phentolamine (Figure 1g) caused a much larger increase of plasma insulin concentration than tolbutamide but this had no effect on aromatic amino acid disposition: plasma, brain and muscle concentrations of tryptophan and phenylalanine were unaltered. The tissue tyrosine concentrations fell but as these changes were not prevented by streptozotocin pretreatment they were probably unrelated to insulin secretion and may have been due to increased tyrosine catabolism as tyrosine aminotransferase is induced by phentolamine (Govier & Lovenberg, 1969).

It appears therefore that only a considerable plasma insulin increase such as might occur after food intake or a considerable insulin deficiency are likely to alter appreciably the disposition of tryptophan and other aromatic amino acids between plasma and brain. Thus decreases in plasma insulin of 50% (streptozotocin, Figure 1c; propranolol) or increases even up to 300% (phentolamine) had little effect. The results with aminophylline appear to contradict the above generalization as 3 h after its injection amino acid disposition was altered even though plasma insulin only increased by about 100%. However the amino acid changes may have resulted from the very much larger increases of plasma insulin found 1 h after aminophylline injection (Curzon & Fernando, 1976).

Other work supports the conclusion that considerable increases of insulin are needed to alter the tissue uptake of aromatic amino acids as plasma insulin had to be raised as high as 200–300 µu/ml in order to double the rate of entry of tryptophan into the rabbit brain (Daniel, Love, Moorhouse & Pratt, 1975). Plasma insulin may well rise comparably on food intake and thus lead to increased brain tryptophan (Fernstrom & Wurtman, 1971, 1972).

An apparent lack of effect of drugs on amino acid disposition even though they altered insulin secretion could occur if they had other effects on amino acid disposition which obscured those of insulin, e.g. effects dependent on the hypothermia due to phentolamine (Tsoucaris-Kupfer & Schmitt, 1972). Results indicated that propranolol or phentolamine-dependent mechanisms other than the effects of these drugs on insulin levels did not obscure the effects of the insulin changes on amino acid disposition i.e. changes of the latter were not revealed when propranolol or phentolamine were given to rats previously treated with the diabetogenic drug streptozotocin. The only exceptions were the small but significant decreases of muscle and brain phenylalanine when phentolamine was given to streptozotocin-treated rats.

In general, the drugs used did not alter brain tryptophan, 5-HT or 5-HIAA concentration. However, there were two exceptions. Firstly, 72 h after injection of streptozotocin (65 mg/kg i.v.) although brain tryptophan fell 5-HT and 5-HIAA remained unaltered which may suggest a concurrent increase of tryptophan hydroxylase activity. Secondly, tolbutamide caused a large increase of 5-HIAA. This appeared to result not from increased 5-HT turnover but from slower efflux of 5-HIAA from the brain. Thus when efflux was blocked by probenecid, tolbutamide caused only a small percentage increase of 5-HIAA which was probably due to a large increase of brain tryptophan that occurred only when both probenecid and tolbutamide were given.

Tolbutamide may well block 5-HIAA efflux from the brain as it is metabolized to *N*-*p*-carboxybenzenesulphonyl $N^1-n$ butylurea (Wittenhagen, Mohnike & Langenbock, 1959) which is structurally similar to probenecid (*p*-di-*n*-propylsulphamyl-benzoic acid). Furthermore another probenecid-like action of tolbutamide has been described as both drugs increase uric acid excretion (Iyer & Cyriac, 1970).

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# SOME OBSERVATIONS ON THE ANORECTIC ACTIVITY OF PROSTAGLANDIN $F_{2\alpha}$

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1 Intracerebroventricular injection of prostanglandin $F_{2\alpha}$ (10–40 μg) decreases food intake in a dose-dependent manner in rats trained to consume their daily total food intake in a 2 h period.

2 This anorexia is also observed in satiated rats, which had *ad libitum* access to food.

3 The anorectic activity of prostaglandin $F_{2\alpha}$ is not modified by changes in the internal environment of the body after food intake, such as increased blood glucose and insulin levels and decreased fatty acid levels, or by the presence or absence of food in the stomach, as is evident from the anorectic activity of prostaglandin $F_{2\alpha}$ in partially satiated rats.

4 The anorexia is not due to pain or irritative properties of prostaglandin $F_{2\alpha}$ since induction of comparable pain with 3% acetic acid does not affect food intake in rats deprived of food for 22 hours.

5 Anorectic doses of prostaglandin $F_{2\alpha}$ when injected intraperitoneally cause hypothermia.

6 The results suggest that the inhibitory activity of prostaglandin $F_{2\alpha}$ on food intake is at both peripheral and central sites.

7 Prostaglandin $F_{2\alpha}$-induced anorexia is associated with the behavioural tranquillization that is seen after the ingestion of food.

## Introduction

Horton (1964) was the first to introduce prostaglandins into the field of appetite regulation. While studying the effect of prostaglandins $E_1$, $E_2$ and $E_3$ on injection into the cerebral ventricles of cats, he observed that they did not cause hyperphagia, but in cats starved for 24 h before injection a disinterest in food was observed. Later experiments with rats trained to press a bar for food demonstrated that various prostaglandins ($E_1$, $E_2$, $F_{1\alpha}$, $F_{2\alpha}$, $A_1$, $B_1$) when given subcutaneously decrease feeding (Scaramuzzi, Baile & Mayer, 1971). Further studies on the possible site of action have shown that injection of prostaglandin $E_1$ into the lateral hypothalamic, anterior commissure, perifornical and ventromedial hypothalamic areas reduced food intake in rats, while prostaglandin $B_1$ injections had no effect (Baile, Bean, Simpson & Jacobs, 1971; Baile, Simpson, Bean, McLaughlin & Jacobs, 1973). These workers also reported that there were no behavioural depressant effects nor was there any change in water intake. Since prostaglandins are interrelated with fatty acids and adipose tissue and since they depress feeding when injected into areas of the hypothalamus concerned with regulation of food intake, they suggested that prostaglandins might be acting as intermediaries between fat depots and the hypothalamus and may be involved in energy balance regulation. Studies involving sheep have revealed a dual action of prostaglandin $E_1$ on food intake. When injected into loci in the medial and anterior hypothalamus where (–)-noradrenaline injections elicited feeding ($\alpha$-adrenoceptor loci), prostaglandin $E_1$ reduced feeding, while injections of prostaglandin $E_1$ into loci in the lateral anterior hypothalamus (which are $\beta$-adrenoceptor sensitive) elicited feeding. These effects were specific for prostaglandin $E_1$ since prostaglandin $E_2$ did not have any effect when injected into those loci. Injection of a prostaglandin antagonist into the $\alpha$-adrenoceptor loci increased feeding when given alone and antagonized the hypophagia induced by prostaglandin $E_1$ on prior injection (Martin, Baile, Webb & Kingsbury, 1972; Baile & Martin, 1973; Baile, Martin, Forbes, Webb & Kingsbury, 1974). Most of the studies mentioned above were concerned with injection of E or B prostaglandins and no detailed studies have been reported with prostaglandin $F_{2\alpha}$ on food intake when injected into the central nervous system. Prostaglandin $F_{2\alpha}$ effects are more relevant in an understanding of the possible physiological role of prostaglandins in this important component of energy balance because prostaglandin $F_{2\alpha}$ is a natural constituent of all the mammalian brains studied (Holmes & Horton, 1968), and furthermore F prostaglandins are the only ones that can be synthesized by rat brain (Feldberg, 1974). A preliminary investigation on food intake when prostaglandin $F_{2\alpha}$ was injected into the cerebroventricular system has been reported earlier (Doggett & Jawaharlal, 1975). The present paper is an extension of our earlier study.

## Methods

### *Animals*

Male albino rats weighing 200–250 g were used in these experiments.

(a) Hungry rats: The method employed in this type of study is the one described earlier (Hollifield & Parson, 1962). Rats were trained to eat all their food in 2 h per day (between 14 h 00 min–16 h 00 min). After 15 days of training when the animals had adapted to the 2 h food regimen as indicated by their positive weight gain, the animals were used for experiment. Rats not adapting to this regimen and showing no weight gain were discarded. Water was allowed *ad libitum*.

(b) Satiated rats: The method is based on that of an earlier study (Grossman, 1962). All the experiments were done at 12 h 00 min, the time when the food intake was found to be minimal in all animals. In this experiment both food and water were allowed *ad libitum*.

### *Laboratory conditions*

All the rats were housed in a room maintained at 25 ± 1°C with a 12 h light/dark cycle, all experiments being performed in the light. Animals were maintained on a conventional 41B cube diet (Spilsburys, Birmingham).

### *Estimation of food intake*

The rats were housed singly in cages with wire grid bottoms. In order to collect the spillage during experiments, paper was placed under the cages and an accurate measure of the amount of food eaten was obtained by reweighing the left-over food together with the spillage. The food intake was measured at regular intervals of 15, 30, 60, 90 and 120 min for rats under 2 h feeding tests and after 1 h for satiated rats. In experiments with satiated rats, in order to ensure satiety conditions the rats were given fresh food 30 min before the experiment started. However, no attempt was made to get super satiety by forced feeding. Three types of controls were maintained: (1) no injection; (2) the needle was inserted into the cannula and removed without injection; (3) injection of vehicle.

The food intake is expressed as the amount eaten in g/100 g body weight in the case of experiments with hungry rats and the total amount consumed per rat in experiments with satiated animals because of the very small quantity of food eaten by these animals. The significance of any observed differences between the group means was determined by Student's *t* test.

### *Implantation of cannula and injection techniques*

The construction of the intracerebroventricular cannula and its implantation was as described by Sparkes & Spencer (1971). At least a week was allowed to elapse between implantation and the start of injections. All injections were made into the left lateral ventricle in a volume of 10 to 20 µl. There was an interval of at least two or three days between any two injections. Prostaglandin solutions were always given alternately with an injection of vehicle in any one animal.

In order to minimize the effect of stress caused by handling and by cerebroventricular injections, the animals were repeatedly handled and mock injections were done many times in order to familiarize the animals with the procedure before actual experiments started.

### *Measurement of body temperature*

The rectal temperature was measured as described previously (Lomax, 1966). The thermistor probe was lubricated with liquid paraffin and inserted into the rectum to a depth of at least 6 cm.

### *Drugs and solutions*

Prostaglandin $F_{2\alpha}$ as tromethamine salt used in this experiment was supplied by Dr J.E. Pike, Upjohn Company, Kalamazoo, Michigan and Professor B. Samuelsson, of Karolinska Institute, Stockholm, Sweden. The stock solutions were prepared in 95% ethanol in concentration of 10 mg/ml and were kept frozen until used. All the prostaglandin solutions were prepared fresh before experiments by diluting with pyrogen-free saline (0.9% w/v NaCl solution) containing 0.2 mg/ml of sodium carbonate. This procedure brought the pH range of injected solution in the present experiments to 6.5 to 7.5. All the doses of prostaglandin $F_{2\alpha}$ given refer to the salt.

## Results

### *Hungry rats*

Intracerebroventricular injections of 10–40 µg prostaglandin $F_{2\alpha}$ immediately before access to food in rats trained to consume their total 24 h food intake over a 2 h period, suppressed food intake in a dose-dependent manner compared to those animals which

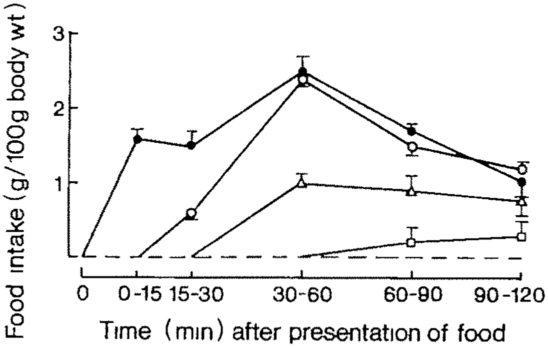

**Figure 1** Effect of intracerebroventricular injection of vehicle (control) (●), 10 μg (○), 20 μg (△) or 40 μg prostaglandin $F_{2\alpha}$ (□) on food intake over a 2 h period in the rat Each point represents the mean of results from 40 rats for control group and 15 rats for groups receiving prostaglandin $F_{2\alpha}$. Vertical lines show s.e. mean. Broken lines indicate complete suppression of food intake.

were injected with vehicle alone or non-injected controls. Since no differences in food intake were observed between vehicle and non-injected groups the results of these were pooled and used as a combined control for comparison with prostaglandin $F_{2\alpha}$-treated rats (Figure 1). Prostaglandin $F_{2\alpha}$ (1 and 5 μg) did not cause any detectable anorexia in the present experimental situation. When a dose of 10 μg was injected it suppressed food intake for only the initial 15 min period of the 2 h feeding session. It was often observed at this dose, that at a later part of the feeding session there was a tendency to compensate for the initial depression, and when bodyweights were measured after 24 h there was no statistically significant difference between the controls and treated group ($P<0.1$). However, a dose of 20 μg which suppressed food intake completely for the first 30 min of the feeding period, and whose anorectic activity was still evident in the latter part of the feeding period, produced a fall in the 24 h bodyweights, when compared to the vehicle-treated group which registered a slight positive gain in weight (Figure 2). The highest dose (40 μg) of prostaglandin $F_{2\alpha}$ used in the present study produced marked anorexia; it completely suppressed food intake for the first hour and even in the final 60 min of the feeding period only a small food intake occurred. Individual variations were observed between rats both in control food intake and its suppression by prostaglandin $F_{2\alpha}$. In some animals there was a quicker recovery from the suppressant activity, while a few exhibited prolonged anorexia. Injection of these doses subcutaneously did not cause anorexia.

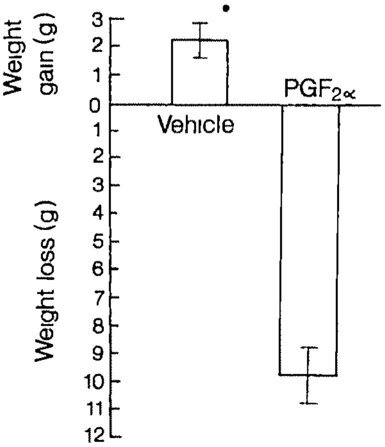

**Figure 2** Effect of intracerebroventricular injection of vehicle or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 20 μg to rats feeding for 2 h period on 24 h body weight. Each column is the mean of results from 15 rats; vertical lines show s.e. mean.

### *Partially satiated rats*

A number of factors can influence the effect of substances on food intake. Changes in the internal environment brought about by a meal, for example increased glucose and insulin levels, have been correlated with food intake (Steffens, 1969; 1970). Furthermore, recent studies have shown that there are differences in the mode of action of the well known anorectic drugs amphetamine and fenfluramine, the former acting upon hunger but the latter on satiety. The consumption of a certain amount of food is required for the inhibitory action of fenfluramine to occur, but this is not so for amphetamine (Blundell, Latham & Lesham, 1976). In the present experiments the effect of these factors were examined on prostaglandin $F_{2\alpha}$-induced anorexia in partially satiated rats. Food was presented to one group of rats for 30 min and to a second group for 60 min of the 2 h feeding period. Injection of prostaglandin $F_{2\alpha}$ (20 μg) intracerebroventricularly suppressed food intake for at least 30 min in both groups (Figure 3), an effect similar to that seen when it was injected before presentation of any food (Figure 1). Since injection of vehicle at these two periods did not affect feeding, the results of both were pooled.

### *Satiated rats*

Experiments were also conducted in satiated animals with access to food *ad libitum*. The effect of injecting 20 μg of prostaglandin $F_{2\alpha}$ which has been shown to suppress food intake for at least 30 min in hungry and partially satiated animals (Figures 1 and 3) was studied and compared with control rats. Figure 4 summarizes the results. In these experimental conditions even when the food intake is minimal prostaglandin $F_{2\alpha}$ still exhibits some anorectic activity.

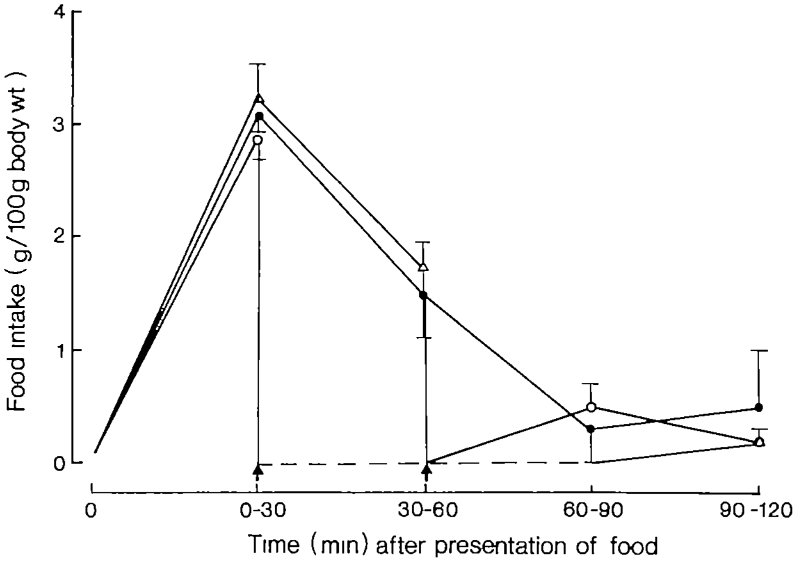

**Figure 3** Effect of intracerebroventricular injection at the arrow of prostaglandin $F_{2\alpha}$ 20 μg 30 min after access to food (○) and of prostaglandin $F_{2\alpha}$ 60 min after access to food (△). Broken lines indicate no eating period. Control group (●). Vertical lines show s.e. mean.

*Involvement of pain in prostaglandin $F_{2\alpha}$-induced anorexia*

Prostaglandins when injected cause pain (Collier & Schneider, 1972), thus raising the possibility that prostaglandin $F_{2\alpha}$-induced anorexia is only secondary to its ability to induce pain. Therefore the effect of similar pain on food intake was studied. Rats deprived of food for 22 h received intraperitoneally vehicle, 1 mg/kg of prostaglandin $F_{2\alpha}$ or 3% acetic acid 5 min before presentation of food. Injection of acetic acid has been shown to produce similar pain (Chernov, Wilson, Fowler & Plummer, 1967) by this route. There was no statistically significant difference in food intake between vehicle and either acetic acid or non-treated animals. Prostaglandin $F_{2\alpha}$ 1 mg/kg by the same route completely suppressed food intake for the initial 30 min (Figure 5).

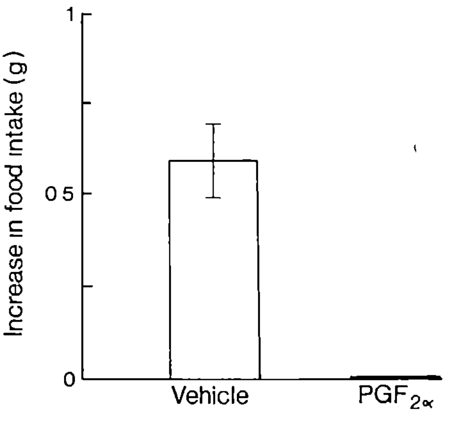

**Figure 4** Effect of intracerebroventricular injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 20 μg or vehicle on food intake in the satiated rat. Each column is the mean of results from 15 rats; vertical lines show s.e. mean.

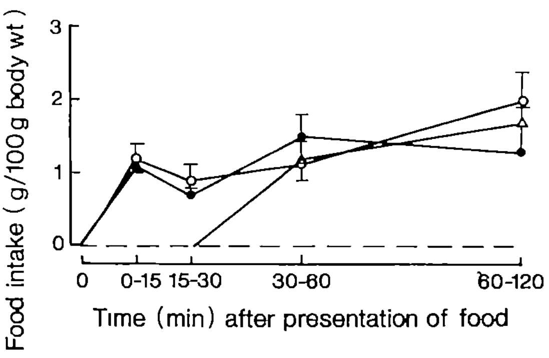

**Figure 5** Intraperitoneal administration of vehicle 1 ml/kg (control) (●), prostaglandin $F_{2\alpha}$ 1 mg/kg (△) or 3% acetic acid 1 ml/kg (○) on food intake in 22 h food deprived rat. Each point represents the mean of results from at least 6 rats; vertical lines show s.e. mean. Broken lines indicate complete suppression of food intake.

### *Effect of prostaglandin $F_{2a}$ on body temperature*

Prostaglandin $F_{2a}$ when injected intracerebroventricularly in rats causes hyperthermia and anorexia (Doggett & Jawaharlal, 1975) so the possibility exists that its anorectic activity is secondary to its hyperthermic effect. Intraperitoneal injection of 0.5 and 1 mg/kg body weight of prostaglandin $F_{2a}$ to rats deprived of food for 22 h produced whole body hypothermia (Figure 6) which was maximal at 90 min after administration.

### *Behavioural effect of prostaglandin $F_{2a}$*

Injection of prostaglandin $F_{2a}$ either intracerebroventricularly or intraperitoneally in food-deprived rats caused no signs of central nervous depression, ptosis or catatonia nor was there any effect on respiration. The enhanced locomotor activity associated with searching for food, restlessness and the aggressive behaviour resulting from food deprivation were all decreased after prostaglandin $F_{2a}$ treatment. The rats showed disinterest in food even when food pellets were placed near them. The 'behavioural tranquillization' that occurs after a meal is the appropriate description for the prostaglandin-treated rat, which becomes behaviourally indistinguishable from the satiated animal.

### *Other effects*

No changes on water intake were observed after prostaglandin $F_{2a}$. Intracerebroventricular injections of this prostaglandin did not induce salivation as in dogs (Hahn & Patil, 1972) or produce gastrointestinal symptoms such as diarrhoea, as in cats (Milton & Wendlandt, 1971).

## Discussion

Studies from a number of laboratories in the last few years (Horton, 1964; Scaramuzzi *et al.*, 1971; Baile *et al.*, 1973; Doggett & Jawaharlal, 1975) have implicated prostaglandins in regulation of food intake in a number of species. Prostaglandins have been shown to have many other actions which affect food intake. They cause irritation and pain (Collier & Schneider, 1972); when injected into the ventricular system of the brain they produce hyperthermia (Milton & Wendlandt, 1971; Doggett & Jawaharlal, 1975). Therefore it is possible that the anorectic activity of prostaglandins is only secondary to those actions. In the present study induction of similar irritation and pain with acetic acid did not affect food intake. Furthermore, in view of the chemospecific and loci-specific activity of prostaglandins when injected into hypothalamic areas, the possibility of pain and irritation as the causes for their anorectic activity can be excluded. A number of other reasons also exclude hyperthermia as the cause of their anorectic activity. There is no correlation between their anorectic and hyperthermic activities (Baile *et al.*, 1973; Doggett & Jawaharlal, 1975). Injection of prostaglandins peripherally cause anorexia and hypothermia (Figure 6). Substances that cause hyperthermia when injected intracerebroventricularly have also been shown to produce 'hyperphagia' (Myers & Yaksh, 1968). However, this does not exclude prostaglandins being the cause of the depressant effect of pyrogens on food intake (Beamer, Thomas & Moore, 1954) since pyrogens increase prostaglandin levels (Feldberg, Gupta, Milton & Wendlandt, 1973). One theory of food intake regulation concerns temperature. This theory assumes that the specific dynamic action of food, which is the cause of increased temperature after a meal, is related to the onset of satiety (Anand, 1961). It has been shown that prostaglandin levels increase after a meal (Greaves, Mcdonald-Gibson & Mcdonald-Gibson, 1972) and whether this release is due to specific dynamic action of food and the cause of increased temperature and the onset of satiety seems an interesting possibility.

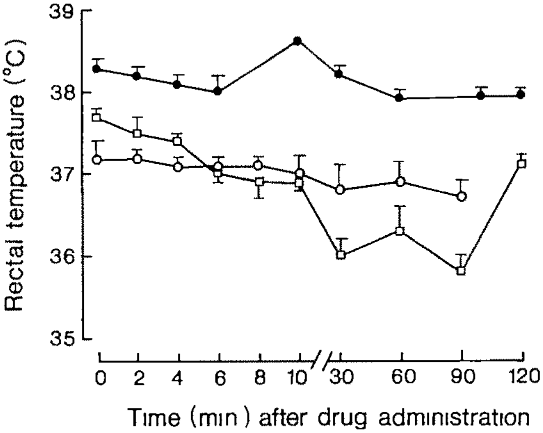

**Figure 6** Effect of intraperitoneal administration of vehicle (control) (●), prostaglandin $F_{2a}$ 0.5 mg/kg (O) or 1 mg/kg (□) on the rectal temperature in 22 h food deprived rat. Each point represents the mean of results from 8 rats. Vertical lines show s.e. mean.

Possible explanations for the anorectic effect were looked for but nothing inconsistent with satiety was found. Practically all the prostaglandins so far studied cause anorexia (see Introduction) with the exception of prostaglandin $E_1$ in certain regions of the hypothalamus in sheep where an increase in food intake was produced (Martin *et al.*, 1972; Baile & Martin, 1973; Baile *et al.*, 1974). However, whether this is a genuine species difference or due to other factors is not clear, because a similar study with prostaglandin $F_{2\alpha}$ has not been made, in spite of it being the commonest prostaglandin found in all the mammalian brains studied (Holmes & Horton, 1968).

From the present results it is interesting to note that the anorectic activity of prostaglandin $F_{2\alpha}$ is not influenced by the changes in the internal environment of the animal, such as an increase in blood glucose and insulin levels and a decrease in free fatty acids. This indicates that prostaglandins probably act on basic mechanisms of food intake regulation and so may act as mediators of both pre- and post-absorptive satiety signals.

It could be postulated that prostaglandins are involved in the physiological satiety mechanism, particularly since they have more than one site of action, both peripheral and central. This is supported by the fact that prostaglandins $B_1$ and $E_2$ injected peripherally depress food intake (Scaramuzzi *et al.*, 1971), while they are devoid of activity when injected into some areas of the hypothalamus, unlike prostaglandin $E_1$ (Baile *et al.*, 1973; Baile *et al.*, 1974). This raises the possibility that the release of specific prostaglandins might mediate satiety at different sites. Release of prostaglandins has been demonstrated after distension of the stomach (Bennet, Friedman & Vane, 1967) and stretching of the stomach itself causes satiety (Anand, 1961). Furthermore, there are other factors from the gastrointestinal tract which induce release of prostaglandins that can contribute to satiety. For example, Vane (1972) suggested that prostaglandins might be released by many stimuli including gentle massage, comparable to the 'massage' induced by gastrointestinal churning of ingested food. Prostaglandins of the E and A series are potent inhibitors of gastric secretion in several species (Main, 1973), where they might alter the pH of the stomach and therefore might be responsible for stimulation of postulated pH receptors of the gastrointestinal system (Sharma, 1967) which participate in the regulation of food intake.

The most important post-obsorptive sites where prostaglandins may have a role are adipose tissue and liver. Since they are released from adipose tissue (Shaw & Ramwell, 1968), and since depot fat plays an important role in the regulation of food intake (Kennedy, 1953), it has been suggested (Baile *et al.*, 1973) that prostaglandins may be the intermediaries between hypothalamic feeding centres and depot fat. However, in view of their effective degradation by lung (Nakano, Morsy & Distler, 1970) the amount that is likely to reach the hypothalamic centres must be small and hence an effect there is unlikely. Since an adipokinetic mechanism has been demonstrated (Panksepp & Pilcher, 1973) within the hypothalamus it is most likely that prostaglandins might be released there and act as local transmitters. Since the liver contains receptors for regulation of food intake (Russek, 1971) and since prostaglandins are accumulated in large quantities (Bergstrom, Carlson & Weeks, 1968) in this organ, this might be their other site of action.

Recent studies have demonstrated that changes in the level of cyclic adenosine 3',5'-monophosphate (cyclic AMP) affect feeding in the rat (Booth, 1972). However, the anorectic action of centrally administered prostaglandin $F_{2\alpha}$ is unlikely to be mediated through this mechanism because it has been shown that this prostaglandin does not affect formation of cyclic AMP in the cerebral cortex of the rat (Berti, Trabucchi, Bernareggi & Fumagalli, 1973).

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# ANORECTIC ACTIVITY OF PROSTAGLANDIN PRECURSORS

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1 Intraperitoneal and intragastric (i.g.) administration of prostaglandin precursors arachidonic (2 mg, 15 mg/kg, i.p.; 30 mg/kg, i.g.), linolenic (100 mg/kg, i.p.; 200 mg/kg, i.g.) and linoleic (15, 100 mg/kg, i.p.; 100 mg/kg, i.g.) acids to 22 h food-deprived rats inhibits food intake.

2 This anorexia is similar to that induced by prostaglandin $F_{2\alpha}$ (1 mg/kg, i.p.).

3 At anorectic doses these fatty acids do not cause pyrexia, in fact arachidonic acid causes hypothermia.

4 Prior treatment with indomethacin (15 mg/kg) and paracetamol (50 mg/kg) specifically reverses the anorexia and the behavioural satiety induced by the three fatty acids, while not affecting prostaglandin $F_{2\alpha}$-induced suppression of food intake.

5 Results of the present experiments suggest that both physiological and pharmacological modification of appetite could be brought about through an effect on prostaglandin generating systems.

## Introduction

Prostaglandins are of ubiquitous distribution within the body and are synthesized from essential fatty acid precursors by various tissues including the brain (Bergstròm, Danielsson, Klenberg & Samuelsson, 1964; Bergström, Danielsson & Samuelsson, 1964). Since the first report (Horton, 1964), their possible involvement in the regulation of the hunger–satiety phenomenon has been studied by a number of workers (for references see Doggett & Jawaharlal, 1977). It has been shown that in man, fasting is associated with lower prostaglandin levels in the blood as compared with post-prandial samples (Greaves, McDonald-Gibson & McDonald-Gibson, 1972). Apart from this, insulin has been shown to play an important role in the hunger–satiety phenomenon (Mayer, 1955) and is closely associated with the formation of polyunsaturated fatty acids, which are prostaglandin precursors. For example, desaturation of fatty acids is markedly depressed in the liver of diabetic animals in a similar way to that produced by fasting (Benjamin & Gellhorn, 1964), an effect which in both cases can be reversed by insulin (Mercuri, Peluffo & Brenner, 1966). Furthermore diabetes mellitus, an insulin deficient state, is characterized by 'hyperphagia'. Earlier studies on the possible role of fatty acids as a satiety signal have not yielded any information as to their possible role in appetite regulation, although their role has been emphasized (Kennedy, 1966). Intravenous infusion of neutral fat emulsions has no effect on the electrical activity of the lateral or ventromedial hypothalamus, both of which play an important part in appetite regulation (Anand, Dua & Singh, 1961). An inverse correlation between non-esterified free fatty acid concentrations and glucose utilization exists which is thus related to hunger–satiety (Mayer, 1963). In rats deprived of food for 1 h, it has been shown that oleic acid produces latent anorexia after intragastric administration (Booth, 1972). A number of esters of saturated fatty acids, like palmitic acid, on feeding for 30 days to rats have recently been shown to increase food intake, feed efficiency and weight gain when they provided 36% of dietary energy (Caster, Resurreccion, Cody, Andrews & Bargmann, 1975). The anorexigenic activity of oleic acid, the monoenoic fatty acid, is very interesting in view of the opposite effect of palmitic acid, since both these fatty acids together constitute approximately 70% of free fatty acids in the rat blood (Oomura, Sugimori, Nakamura & Yamada, 1975). In view of the anorexigenic activity of the prostaglandins, a study of the possible role of their precursor polyunsaturated fatty acids in this homeostatic mechanism is warranted: this is the subject of the present paper.

## Methods

### *Measurement of food intake*

Male albino rats weighing between 200–250 g were housed singly in cages with wire grid bottoms in which they were allowed to acclimatize for 3 days with free access to food and water. During experiment water was available *ad libitum* throughout, but the animals were deprived of all food for 22 h before presentation of 100 g 41B pellets. Since rats eat mainly at night (Richter, 1927; Hemmingsen & Krarup, 1937), all experiments were performed in darkness between 18 h 00 min and 24 h 00 min, the artificial lighting being extinguished 1 h before the start. Accurate values of food intake were obtained by adding the spillage collected on the papers placed underneath the cages during experiments to the leftover food while reweighing. Food intake was measured at 15 min intervals up to 2 h and expressed as the mean weight eaten per 100 g body weight. At least 7 days elapsed between consecutive experiments in the same animal. The significance of any observed differences between the group means was determined by Student's *t* test.

### *Intragastric administration*

Intragastric administration was accomplished with a stainless steel hypodermic needle beaded at the end and curved to facilitate easy oesophageal insertion. The procedure was repeated 2 or 3 times before the actual experiment in order to accustom the rat to the procedure.

### *Measurement of the body temperature*

The rectal temperature was measured by the method described previously (Lomax, 1966). The thermistor probe was lubricated with liquid paraffin, and inserted into the rectum to a depth of at least 6 cm.

### *Drugs and solutions*

The following substances were used: arachidonic acid sodium salt, linoleic acid sodium salt, linolenic acid ethylester, indomethacin and paracetamol.

Prostaglandin $F_{2\alpha}$ as tromethamine salt (supplied by Dr J.E. Pike, Upjohn Co., U.S.A. and Professor B. Samuelsson, Karolinska Institute, Stockholm,

**Table 1** Effect of intraperitoneal administration of fatty acids on food intake in 22 h fasted rats

| *No.* | *Fatty acid* | *Dose (mg/kg, i.p.)* | *Time of administration of fatty acid before presentation of food* | *Food intake (g/100 g body wt.)* | |
|---|---|---|---|---|---|
| | | | | *0–30 min* | *0–60 min* |
| I | Tween-80-saline | | 5 min | | 2.64 ± 0.44 |
| (a) | Arachidonic acid | 15 | 5 min | | 0.66 ± 0.22<br>($P<0.01$) |
| (b) | Tween-80-saline | | 15 min | | 4.88 ± 0.91 |
| | Arachidonic acid | 15 | 15 min | | 0.62 ± 0.24<br>($P<0.01$) |
| (c) | Tween-80-saline | | 30 min | 3.14 ± 0.30 | 3.95 ± 0.57 |
| | Arachidonic acid | 15 | 30 min | 1.21 ± 0.34<br>($P<0.01$) | 2.84 ± 0.57<br>(NS) |
| II | Tween-80-saline | | 5 min | | 3.02 ± 0.31 |
| (a) | Linoleic acid | 100 | 5 min | | 1.42 ± 0.06<br>($P<0.01$) |
| (b) | Tween-80-saline | | 15 min | | 4.88 ± 0.91 |
| | Linoleic acid | 100 | 15 min | | 1.52 ± 0.31<br>($P<0.05$) |
| (c) | Tween-80-saline | | 30 min | | 2.60 ± 0.40 |
| | Linoleic acid | 100 | 30 min | | – |
| III | Arachis oil | | 30 min | 1.66 ± 0.23 | 2.60 ± 0.30 |
| (a) | Linolenic acid | 100 | 30 min | 0.45 ± 0.30<br>($P<0.01$) | 2.08 ± 0.47<br>(NS) |

Values are mean ± s.e. mean. $n$ = at least 4.

Sweden) was prepared freshly from a stock ethanolic solution (10 mg/ml) with pyrogen-free saline (0.9% w/v NaCl solution) containing 0.02% sodium carbonate. Arachidonic and linoleic acids were dissolved in a solution of 0.5% Tween 80 in saline, while linolenic acid was dissolved in arachis oil. Paracetamol was prepared in hot saline containing 20% propylene glycol as described by Milton & Wendlandt (1971). Indomethacin was suspended in 0.5% methylcellulose in water.

Indomethacin was given subcutaneously; all other substances were administered intraperitoneally or intragastrically. The volume of injection was 1 ml/kg in all cases except for paracetamol which was 2 ml/kg. In order to study the effect of prostaglandin biosynthesis blockers on hunger, both indomethacin and paracetamol were administered 1 h before each of the fatty acids. Food was presented 5 min after the administration of fatty acids.

## Results

### *Anorectic activity of prostaglandin precursors*

Table 1 summarizes the effect of the three fatty acids administered intraperitoneally on food intake in rats deprived of food for 22 hours. The onset of anorexia after both arachidonic acid and linoleic acid occurred within a few minutes of administration. Anorectic activity of arachidonic acid appears to last for at least 60 min. With linoleic acid, reduction of food intake occurred 30 min after administration of the fatty acid. The fact that linoleic acid has to be further converted to arachidonic or linolenic acid, while linolenic acid and arachidonic acid are the immediate precursors of prostaglandins, could account for this. Intragastric administration (Table 2) of the three fatty acids also caused anorexia. All the three fatty acids produced a statistically significant decrease in food intake in the next hour when food was presented 30 min after fatty acid treatment, while only arachidonic acid still caused a statistically significant reduction in intake of food offered 60 min after treatment.

### *Prostaglandin synthesis blockers on anorectic activity of fatty acids and prostaglandin $F_{2\alpha}$*

In Figure 1, the effect of prostaglandin synthetase inhibitors or vehicle alone on arachidonic acid (2 mg/kg)-induced suppression of food intake is depicted. In vehicle pretreated rats, arachidonic acid caused complete suppression of food intake for the first 15 min after presentation of food. Indomethacin

**Table 2** Effect of intragastric administration of fatty acids on food intake in 22 h fasted rats

| | | | *Time of administration of fatty acid before presentation of food* | *Food intake (g/100 g body wt.)* | |
|---|---|---|---|---|---|
| | *Fatty acid* | *Dose* | | *0–30 min* | *0–60 min* |
| I | Control (Tween-80-saline) | 1 ml/kg | | | 4.64 ± 0.58 |
| | Arachidonic acid | 30 mg/kg | 30 min | | 1.90 ± 0.67<br>($P < 0.02$) |
| | Arachidonic acid | 30 mg/kg | 60 min | | 1.47 ± 0.70<br>($P < 0.01$) |
| II | Control (Tween-80-saline) | 1 ml/kg | | 2.79 ± 0.36 | 4.88 ± 0.74 |
| | Linoleic acid | 100 mg/kg | 15 min | 0.24 ± 0.24<br>($P < 0.01$) | 1.47 ± 0.28<br>($P < 0.01$) |
| | Linoleic acid | 100 mg/kg | 30 min | 0.67 ± 0.36<br>($P < 0.01$) | 2.71 ± 0.50<br>($P < 0.05$) |
| | Linoleic acid | 100 mg/kg | 60 min | 1.89 ± 0.06<br>($P < 0.05$) | 4.70 ± 0.38<br>($P < 0.1$) |
| III | Control (Arachis oil) | 1 mg/kg | | 2.20 ± 0.42 | 3.50 ± 0.61 |
| | Linolenic acid | 200 mg/kg | 30 min | 0.21 ± 0.21<br>($P < 0.01$) | 1.49 ± 0.77<br>($P < 0.05$) |
| | Linolenic acid | 200 mg/kg | 60 min | 1.09 ± 0.34<br>($P < 0.05$) | 2.96 ± 0.58<br>($P < 0.1$) |

Values are mean ± s.e. mean. $n$ = at least 4.

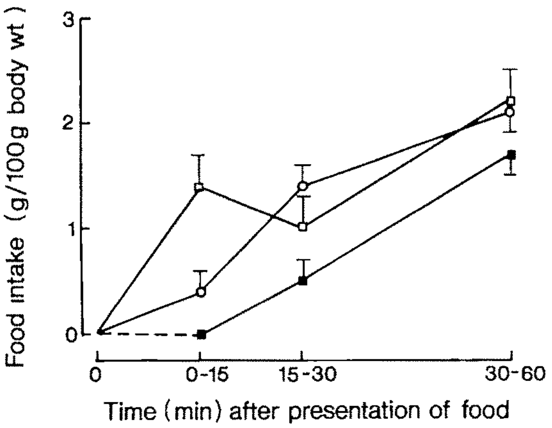

**Figure 1** Effect of 1 h pre-treatment with vehicle (■), indomethacin (□, 15 mg/kg s.c.), and paracetamol (○, 50 mg/kg, i.p.) on suppression of food intake induced by arachidonic acid (2 mg/kg, i.p.) in 22 h food-deprived rats. Food was presented 5 min after administration of fatty acid. Each point represents the mean of results from at least 4 rats. Vertical lines show s e mean.

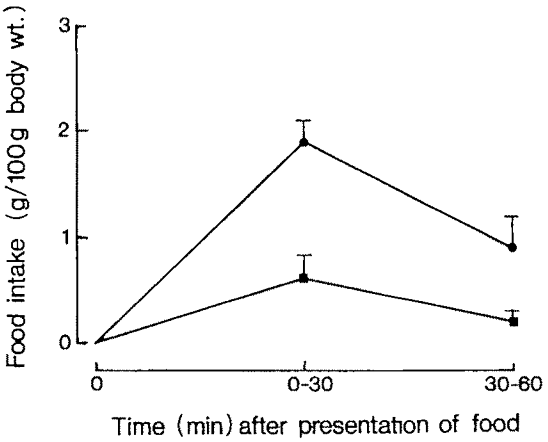

**Figure 2** Effect of 1 h pre-treatment with vehicle (■), and paracetamol (●, 50 mg/kg, i p.) on suppression of food intake induced by linoleic acid (15 mg/kg, i.p.) in 22 h food-deprived rats. Food was presented 5 min after administration of fatty acid. Each point represents the mean of results from at least 4 rats. Vertical lines show s.e. mean.

(15 mg/kg) completely reversed this anorectic activity, immediately after presentation of food. The effect of paracetamol in the first 15 min was weak, but its activity then became much more evident. At the end of the 1 h feeding period those rats treated with indomethacin and paracetamol ate significantly more ($P < 0.01$) than the vehicle-treated rats. Although there was an initial latent period in the paracetamol-treated animals in antagonizing the effect of arachidonic acid, such a latency was not seen when linoleic acid (15 mg/kg)-induced anorexia was reversed (Figure 2). None of the blockers antagonized the anorexia caused by prostaglandin $F_{2\alpha}$ (Table 3). Since administration of either blocker alone had no significant effect on food intake compared to vehicle or non-injected rats, the results are not included.

**Table 3** Effect of administration of prostaglandin biosynthesis blockers, indomethacin (subcutaneously) and paracetamol (intraperitoneally) on food intake of 22 h fasted rats

| | Drugs | Dose/route | *Time of administration of the drug before injection of $PGF_{2\alpha}$* | *Food intake (g/100 g body wt.)* 0–30 min | 0–60 min |
|---|---|---|---|---|---|
| I | | | | | |
| (a) | Vehicle (methyl cellulose 0.5%) | 1 ml/kg s.c. | 1 h | 0 | 1.32 ± 0.23 |
| (b) | Indomethacin | 15 mg/kg s.c. | 1 h | 0 | 1.20 ± 0.19 (NS) |
| II | | | | | |
| (a) | Vehicle (propylene-glycol-saline) | 2 ml/kg i.p | 1 h | 0 | 1.36 ± 0.44 |
| (b) | Paracetamol | 50 mg/kg i.p | 1 h | 0 | 1.24 ± 0 34 (NS) |

Values are mean ± s.e. mean. $n$ = at least 4.
NS = not significantly different ($P < 0.1$) from controls.

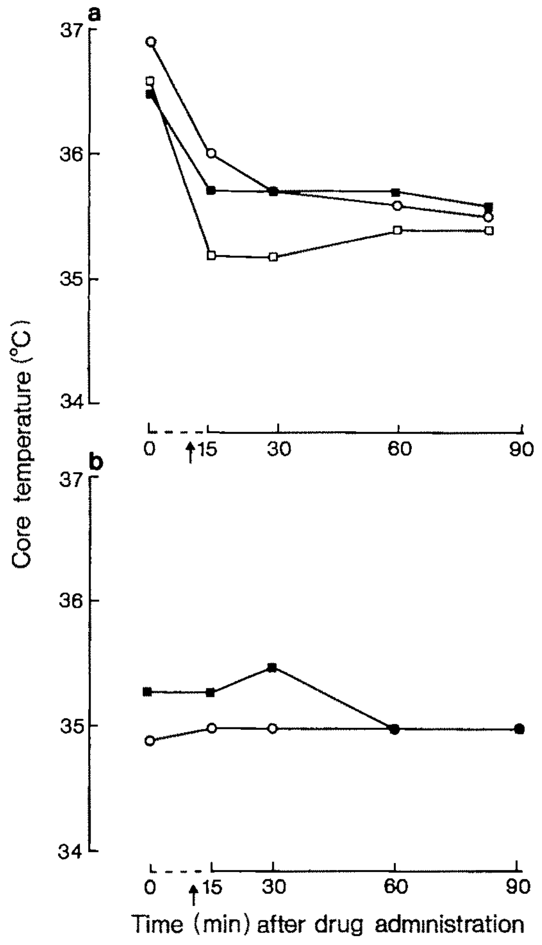

**Figure 3** (a) Effect of intraperitoneal administration of vehicle (■), arachidonic acid (□, 15 mg/kg, i.p.) and linoleic acid (○, 100 mg/kg, i.p.) on the rectal temperature in 22 h food-deprived rats. Each point represents the mean of results from 6 animals. (b) Effect of intraperitoneal administration of arachis oil (■, 1 ml/kg) and linolenic acid (○, 100 mg/kg) on the rectal temperature in 22 h food-deprived rats. Each point represents the mean of results from 6 animals.

### *Behavioural effects of fatty acids, prostaglandin $F_{2\alpha}$ and prostaglandin biosynthesis inhibitors*

All three fatty acids, like prostaglandin $F_{2\alpha}$, at doses which have been shown to cause anorexia, did not produce any central nervous depression nor did they affect locomotor activity. They all caused the typical behavioural tranquillization that is seen after food intake in a food-deprived animal and the animals showed no interest in food. No other behavioural effects were seen. Indomethacin and paracetamol by themselves did not cause any observable behavioural effects. However, they did antagonize the behavioural tranquillization seen after fatty acid administration, and they restored the animals' desire to eat in a similar way to that seen in food-deprived rats.

### *Effect of fatty acids on core temperature*

Measurement of rectal temperature in 22 h food-deprived rats at the highest doses of fatty acids used in this present study indicate complete absence of any pyrogenic activity. In fact, contrary to its hyperthermic effect after intracerebroventricular administration (Splawinski, Gorka, Sudar & Kaluza, 1975), arachidonic acid caused a slight fall in rectal temperature (Figure 3a,b).

## Discussion

The results of the experiments described here clearly indicate that, like prostaglandins, all the three precursor fatty acids possess anorectic activity in food-deprived rats, when given either intraperitoneally or intragastrically before presentation of food. This anorectic activity is blocked by two prostaglandin synthetase inhibitors, indomethacin and paracetamol. Since these fatty acids produce pain (abdominal constrictions) when given intraperitoneally and this pain can be blocked by prostaglandin synthetase inhibitors (Vane, 1973), it is possible that inhibition of the anorectic activity of precursor fatty acids by these drugs is only secondary to their antagonism of such pain. However, this possibility can be excluded since they also cause anorexia by other routes of administration, e.g. intragastric. Furthermore, induction of similar pain with acetic acid does not affect food intake (Doggett & Jawaharlal, 1977). Since these fatty acids do not cause pyrexia an elevation of body temperature can also be excluded as a cause of anorexia. The food-deprived and fatty acid-treated rats and satiated non-treated animals both showed similar behavioural tranquillization.

The specific inhibition by prostaglandin synthetase inhibitors of the anorectic activity of the fatty acids, in the absence of any effect on prostaglandin $F_{2\alpha}$ itself, suggests that the fatty acids themselves do not cause anorexia; rather they have to be converted to prostaglandins. If such an endogenous prostaglandin formation exists and affects food intake, then drugs which inhibit such a formation might be expected to increase food intake, and it was surprising that the two prostaglandin synthetase inhibitors had no effect on food intake when given alone. To this extent our hypothesis is not supported by our findings. However, there are several possible explanations for this lack of effect, including the use of wrong doses of synthetase inhibitors or wrong locations and time. Alternatively, since it has already been shown that prostaglandin formation is low in hungry rats (Greaves *et al.*, 1972), a finding which itself supports the involvement of a prostaglandin generating system in the regulation of the hunger–satiety phenomenon, it could be that in our food-deprived animals endogenous prostaglandin formation was already so low as to be incapable of further reduction. It has also been shown that indomethacin and aspirin, apart from their actions on prostaglandin synthetase (Vane, 1971), inhibit prostaglandin dehydrogenase (Flower, 1974) thereby inhibiting prostaglandin catabolism and contributing to the anorexia often reported as one of the effects of intoxication of these antipyretic analgesics (Woodbury, 1970). However, drugs which specifically inhibit prostaglandin biosynthesis without inhibiting prostaglandin catabolism, for example the acetylenic analogue of arachidonic acid, eicosa 5,8,11,14-tetraynoic acid, should increase food intake. Support for the involvement of a prostaglandin generating system in hunger–satiety can also be cited from the literature. For example, gold thioglucose (Brecher & Waxler, 1949) and bipiperidyl mustard (Rutman, Lewis & Bloomer, 1966) have been shown to cause hyperphagia resulting in obesity in experimental animals, an effect which can be antagonized by prior treatment with indomethacin (Caffyn, 1972). In addition, a wide variety of clinically used drugs, for example psychotropics (Bainbridge, 1968), local anaesthetics (Epstein, 1960), $\Delta^8$-tetrahydrocannabinol (Rating, Broerman, Honecker, Kluwe & Coper, 1972) have been shown to increase food intake; although they are chemically diverse, all inhibit prostaglandin biosynthesis *in vitro* (Bradley, Samuels & Shaw, 1969; Burstein & Raz, 1972; Kunze, Bohn, Kurz & Vogt, 1973; Kunze, Bohn & Bahrke, 1975). Similarly, the anorectic activity of oleic acid (Booth, 1972) and methylxanthines (Fajardo, 1974) can possibly be explained by their *in vitro* inhibitory action on prostaglandin dehydrogenase (Flower, 1974). However, it is difficult to establish a direct causal relationship.

Regarding the site of such a hypothetical prostaglandin generating system one can only speculate. In view of the short latency of anorectic activity it is possible that there are peripheral sites of action, particularly since labelled linoleic acid has been shown to accumulate in liver and adipose tissue (Panksepp, 1975), areas which have been shown to contain receptors that participate in the regulation of the hunger–satiety phenomenon (Russek, 1971). Evidence for a prostaglandin generating system at the hypothalamic level is that the precursor fatty acids cross the blood brain barrier (Alfin-Slater & Aftergood, 1968) and that hypothalamic centres concerned with regulation of food intake have been demonstrated to accumulate labelled linoleic acid when given intragastrically (Panksepp, 1975). Since paracetamol is a specific inhibitor of brain prostaglandin synthetase (Flower & Vane, 1972), the longer time lag in reversing anorectic activity of arachidonic acid shown in this study (Figure 1) can be explained easily.

The present results suggest why Anand, Dua & Singh (1961) failed to find any meaningful correlation when they infused neutral fat emulsions intravenously. It is not the total non-esterified fatty acids that are important, but the individual fatty acid fractions that might determine the formation of prostaglandins at any given time. It has been suggested that one of the factors that regulate biosynthesis of prostaglandins is the presence or absence of other fatty acids that compete for the same enzyme system (Lands, Letellier, Rome & Vanderhoek, 1973).

Is there any pathological state where there is derangement of prostaglandin generating systems and resultant hyperphagia? Although no direct evidence is available, one condition where such possibility appears to be more likely is diabetes mellitus. In this condition there is depression of desaturation of fatty acids due to lack of insulin (Benjamin & Gellhorn, 1964) and consequently a lack of availability of precursors which might limit the formation of prostaglandins. In this condition, administration of exogenous arachidonic acid has been shown to correct the atrophy of the seminal vesicles and testes (Brenner, Peluffo, Mercuri & Restelli, 1968). It would be of interest to know whether the hyperphagia associated with this condition can be controlled by the addition of polyunsaturated fatty acids to the diet of such patients.

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# THE EFFECT OF CLOPROSTENOL ON HUMAN LUTEAL STEROID AND PROSTAGLANDIN SECRETION *in vitro*

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1 Human luteal tissue slices from days 18, 21 and 25 of the menstrual cycle were superfused *in vitro* with Medium 199 alone or containing cloprostenol (1 μg/ml). Concentrations of progesterone, oestradiol-17$\beta$ and prostaglandins $F_{2\alpha}$ and $E_2$ were determined in the superfusate samples.

2 Secretion of steroids and prostaglandins was maintained at an approximately constant level throughout the experiments (21 h in one case) when the tissue was perfused with M199 alone.

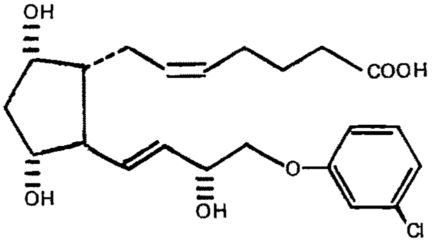
3 Superfusion with cloprostenol (1 μg/ml) resulted in an initial depression of progesterone and oestradiol-17$\beta$ but this was not maintained, levels returning to control values or showing an increase, while superfusion with cloprostenol continued. Cloprostenol is not therefore considered to be luteolytic at this dose and under these conditions for human luteal tissue *in vitro*.

4 Superfusion with cloprostenol (1 μg/ml) also resulted in a large stimulation of secretion of endogenous prostaglandin $F_{2\alpha}$ following a short lag phase. This stimulation was possibly due to the initial depression of progesterone secretion. A short-lived stimulation of prostaglandin $E_2$ secretion was also observed.

5 The significance of the increase in prostaglandin $E_2$ secretion and the interrelationships between the various changes observed with cloprostenol are difficult to interpret.

## Introduction

Luteal function in many mammalian species appears to be controlled by the secretion of a uterine luteolysin which, in most cases, is almost certainly prostaglandin $F_{2\alpha}$ (Poyser, 1973). However, human luteal function seems to be independent of uterine control since neither hysterectomy (Beavis, Brown & Smith, 1969; Beling, Marcus & Markham, 1970) nor congenital absence of Fallopian tubes, uterus and vagina (Brown, Kellar & Matthew, 1959; Fraser, Baird, Hobson, Michie & Hunter, 1973) disrupt cyclical ovarian function. Attempts to induce luteal regression in women by intravenous infusions of prostaglandin $F_{2\alpha}$ have been unsuccessful (Jewelewicz, Cantor, Dyrenfurth, Warren & Vande Wiele, 1972) but direct injection of prostaglandin $F_{2\alpha}$ into the human corpus luteum does induce luteolysis (Korda, Shutt, Smith, Shearman & Lyneham, 1975) and specific prostaglandin $F_{2\alpha}$ receptors have been found in the human corpus luteum (Powell, Hammarstrom, Samuelsson & Sjoberg, 1974). Consequently an intraovarian luteolytic role for prostaglandin $F_{2\alpha}$ has been proposed for the human corpus luteum. The observations that human luteal tissue produces prostaglandins *in vitro* (Challis, Calder, Dilley, Forster, Hillier, Hunter, Mackenzie & Thorburn, 1976a) and that infusions of prostaglandin $F_{2\alpha}$ reduce human luteal progesterone secretion *in vitro* (McNatty, Henderson & Sawyers, 1975) support this hypothesis.



Cloprostenol (ICI 80,996), a derivative of prostaglandin $F_{2\alpha}$, has been shown to be a potent luteolytic agent in several species including sheep, rats and mares (Dukes, Russell & Walpole, 1974). In this paper we present the preliminary results of an investigation into the action of this compound on human luteal steroid and prostaglandin secretion *in vitro*.

## Methods

### *Source of tissue*

Ovarian tissue samples were obtained at laparotomy on days 18, 21 and 25 in 28 day menstrual cycles, from 3 women undergoing hysterectomy for non-malignant conditions (fibroids or endometriosis). The stage of the menstrual cycle was determined from the menstrual history and supported by ovarian and endometrial histology. The project was accepted by the Regional Ethical Committee.

### *Treatment of tissue*

Ovarian tissue was transported to our laboratories in a sterile container immediately on removal from the patient. On arrival in the laboratory the corpora lutea were placed in sterile medium (M199 Wellcome Reagents Ltd.) at pH 7.4. The medium contained no antibiotics or serum at this or any other stage in the experiment. The corpora lutea were then trimmed free of connective tissue and 0.4 mm slices were obtained with a hand microtome. These tissue slices were each then halved and each half slice (50 mg) was accurately weighed and placed in a sterile superfusion chamber. The superfusion system used was as described before (Rodway, Dodson & Watson, 1976) except that the Millipore filters were replaced by nylon gauze. The whole system was set up in a cold room at 4°C, in a heated water bath maintaining the coils, superfusion chambers and contents at 37°C. The apparatus was sterilized 12 h before use by pumping through a 5% (v/v) solution of sodium hypochlorite. Two hours before use the apparatus was rinsed through with distilled water for 1 h and with M199 for the remaining time. Retention of radioactive steroids and prostaglandins by the perspex chambers and the polyethylene tubing was found to be negligible.

In every experiment two blank chambers were set up containing no tissue. Two control chambers containing human luteal tissue were also set up and these and the blank chambers were superfused with M199 alone throughout the experiment. Two experimental chambers containing human luteal tissue were also set up. Initially these chambers were superfused with M199 to allow collection of control samples. Subsequently superfusion was continued with M199 containing cloprostenol (1 μg/ml). After this experimental period, which varied in duration and frequency of sample collection for different experiments, superfusion was again carried out with M199 alone. The duration of sample collection was the same for blank, control and experimental chambers for any given experiment. Also in every experiment a preliminary 90 min superfusion of blank, control and experimental chambers was carried out with M199 and the samples collected during this time were discarded. A flow rate of 20 ml M199/h was used in all experiments for all chambers. All samples were stored at −20°C until extracted and assayed.

### *Analysis of samples*

All samples were analysed for progesterone, oestradiol-17$\beta$, prostaglandin $F_{2\alpha}$ and prostaglandin $E_2$ by radioimmunoassay in each case. All samples were checked for parallelism with the standard curve in every assay and at least three aliquot volumes were assayed in duplicate for every sample.

Tritiated steroid and prostaglandins (Radiochemical Centre, Amersham) had the following specific activities: [$^3$H]-progesterone 255 mCi/mg, [$^3$H]-oestradiol-17$\beta$ 344 mCi/mg, [$^3$H]-prostaglandin $F_{2\alpha}$ 452 mCi/mg and [$^3$H]-prostaglandin $B_2$ 288 mCi/mg.

Quality controls were made up in each case (at a concentration of 5 ng/ml) on the day of the experiment, so that the quality control had been stored for the same length of time as the experimental samples.

The cross-reactivity of cloprostenol was checked in all assays for progesterone, oestradiol-17$\beta$, prostaglandin $F_{2\alpha}$, and prostaglandin $E_2$ and was in all cases less than 0.001% at 50% binding of the antibody. No displacement of the standard curves for any of the above compounds was noted in the presence of cloprostenol at a concentration of 1 μg/ml.

*Progesterone assay.* Samples were extracted and assayed for progesterone by methods previously described (Patek & Watson, 1976), within three weeks of the experiment, where possible. Blanks gave increasing values and quality controls gave variable values if samples were stored for longer than eight weeks. Where analysis was carried out within this period the interassay coefficient of variation, calculated from the quality controls was 6.2% the mean quality control value being 4.72 ng/ml. The intraassay coefficient of variation was 4.6%. Blank sample values were always <5 pg progesterone/ml M199 and it was not considered necessary therefore to adjust the values obtained for control and experimental samples. All samples showed parallelism with the progesterone standard curve.

*Oestradiol-17β assay.* Sample aliquots of various volumes were extracted twice with three volumes of redistilled diethyl ether. Recovery of tritiated oestradiol-17$\beta$ using this extraction method was 86–88%. The dried extracts were assayed by radioimmunoassay using a slight modification of the method of Wu & Lundy (1971). Samples could not be stored for longer than eight weeks without disruption of blank and quality control values. Where analysis

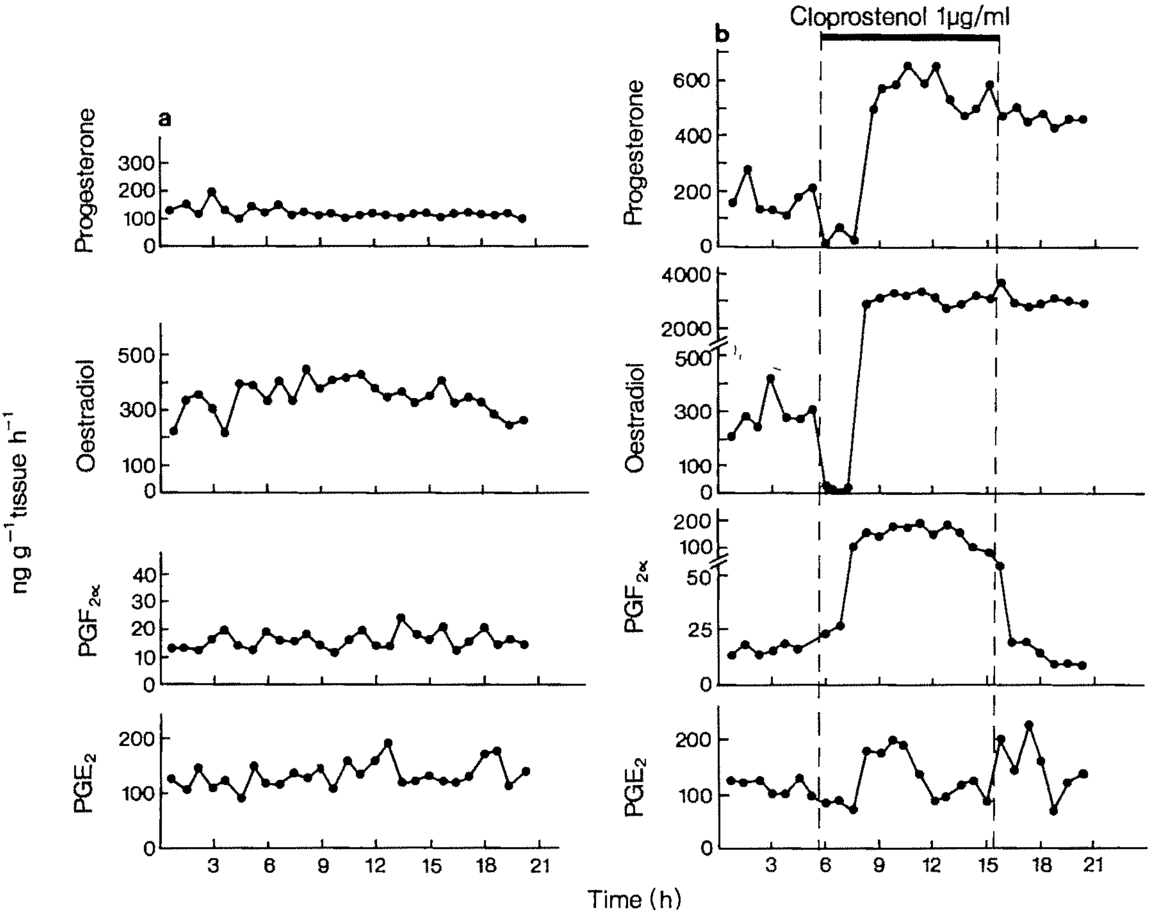


**Figure 1** Day 18 corpus luteum. (a) Mean amounts of steroids and prostaglandins in consecutive samples of superfusate from two control chambers containing tissue superfused with M199 *in vitro*. (b) Mean amounts of steroids and prostaglandins in consecutive samples of superfusate from two chambers containing tissue superfused with M199 or cloprostenol in M199 (horizontal bar) *in vitro*.

was carried out within this time the interassay coefficient of variation was 7.9%, the mean quality control value being 5.53 ng/ml. The intra-assay coefficient of variation was 5.5%. Blank sample values were always between 30 and 50 pg oestradiol-17$\beta$/ml M199 and quality controls, control and experimental samples were adjusted appropriately. All samples showed parallelism to the oestradiol-17$\beta$ standard curve.

*Prostaglandin $F_{2\alpha}$ assay.* Sample aliquots of various volumes (20–200 μl) were acidified with equal volumes of pH 4.0 citrate buffer and extracted twice with two volumes or redistilled diethyl ether. Recovery of tritiated prostaglandin $F_{2\alpha}$ with this extraction method was greater than 95%. The dried extracts were assayed by solid-phased radioimmunoassay by methods previously described (Dighe, Emslie, Henderson, Rutherford & Simon, 1975). Blanks and quality controls were found to store well at −20°C in terms of prostaglandin $F_{2\alpha}$ but all samples were extracted and analysed as quickly as possible, none the less. The interassay coefficient of variation was 5.7%, the mean quality control value being 4.81 ng/ml. The intraassay coefficient of variation was 4.1%. Blank sample values were always less than 1 pg prostaglandin $F_{2\alpha}$/ml M199 and were therefore disregarded. All samples showed parallelism with the prostaglandin $F_{2\alpha}$ standard curve.

*Prostaglandin $E_2$ assay.* All sample aliquots were extracted as soon as possible by the method described for prostaglandin $F_{2\alpha}$. Recovery of tritiated prostaglandin $E_2$ by this extraction method was 95–98%.

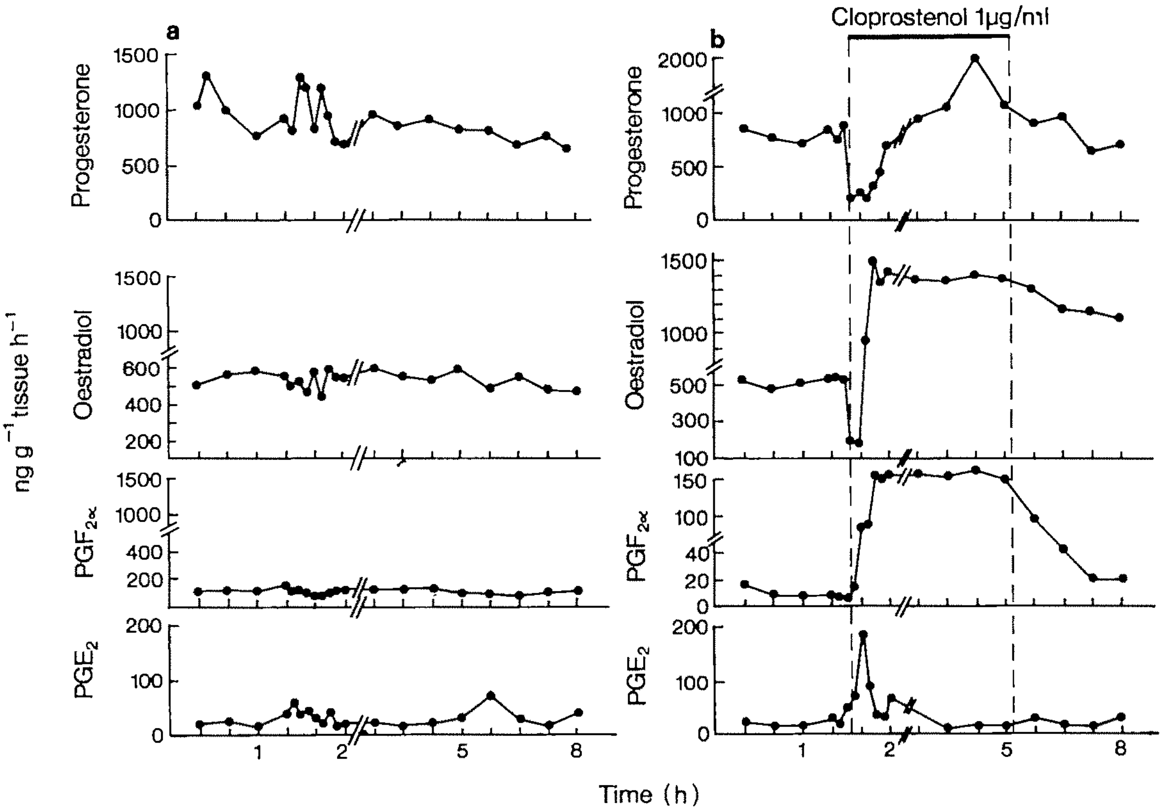


**Figure 2** Day 21 corpus luteum. (a) Mean amounts of steroids and prostaglandins in consecutive samples of superfusate from two control chambers containing tissue superfused with M199 *in vitro*. Samples were collected at 20 min intervals for 80 min, 5 min intervals for the next 40 min and 45 min intervals thereafter. (b) Mean amounts of steroids and prostaglandins in consecutive samples of superfusate from two chambers containing tissue superfused with M199 or cloprostenol in M199 (horizontal bar) *in vitro*. Samples were collected at 20 min intervals for 80 min, 5 min intervals for the next 40 min and 45 min intervals thereafter.

Prostaglandin $E_2$ was assayed as prostaglandin $B_2$ equivalents, the conversion of prostaglandin $E_2$ to prostaglandin $B_2$ being carried out by the method of Dozois & Thompson (1974). Efficiency of conversion was found to average 86% ± 4.6% (mean ± s.e. mean). Conversion was carried out no later than the day following the experiment. Quality controls were treated exactly as the samples. After conversion, prostaglandin $B_2$ was estimated by radioimmunoassay by the method described for prostaglandin $F_{2\alpha}$ by Patek & Watson (1976) except that the antibody used had been raised against prostaglandin $B_2$-bovine serum albumin. All converted quality controls and samples showed parallelism with the prostaglandin $B_2$ standard curve. The prostaglandin $B_2$ content of the quality controls and samples without conversion was also determined and was always less than 5 pg prostaglandin $B_2$/ml M199. The interassay coefficient of variation for quality controls after conversion was 8.2%, the mean quality control value being 4.22 ng/ml the intraassay coefficient of variation for samples after conversion was 7.6%.

## Results

All results quoted are the mean values for samples from two superfusion chambers each containing luteal tissue and each maintained under the same regime.

The patterns of secretion of steroids and prostaglandins for human luteal tissue superfused *in vitro* with M199 alone throughout the experiment are shown in Figures 1a, 2a and 3a for days 18, 21 and 25 of the menstrual cycle, respectively. Figures 1b, 2b and 3b show the patterns of secretion of steroids and prostaglandins for the same tissues, for days 18, 21 and 25 of the menstrual cycle respectively, when superfused with cloprostenol (1 µg/ml) in M199.

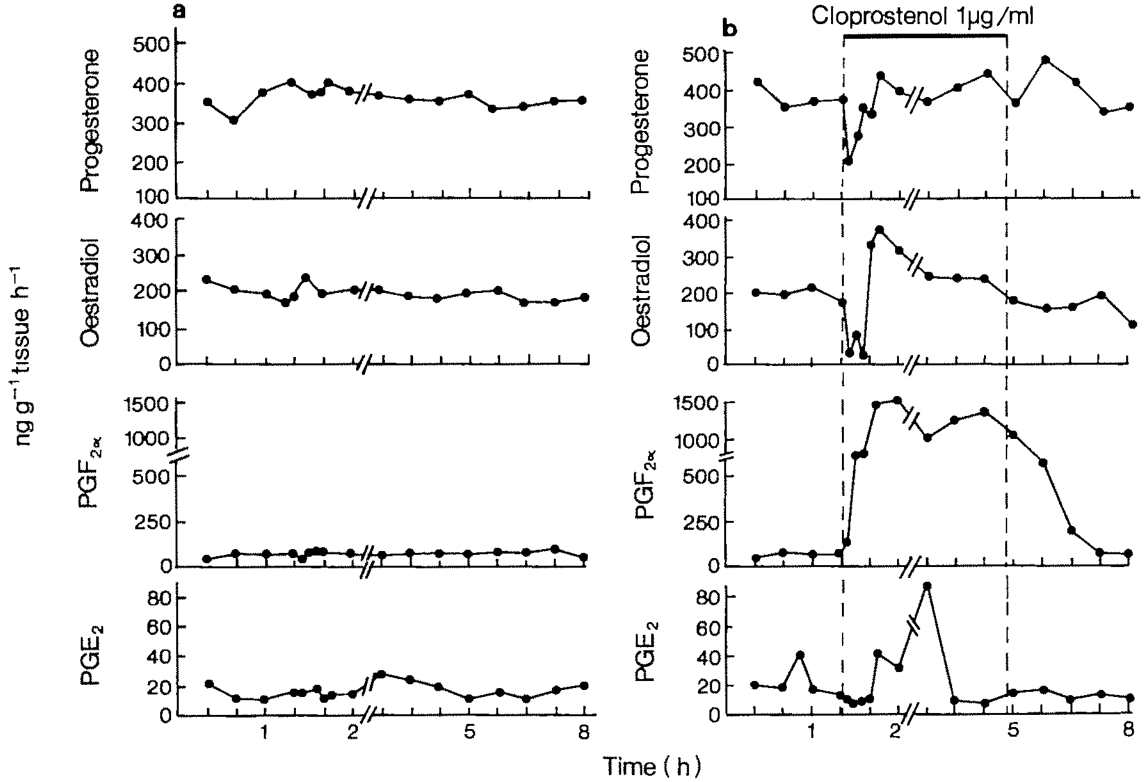


**Figure 3** Day 25 corpus luteum. (a) Mean amounts of steroids and prostaglandins in consecutive samples of superfusate from two control chambers containing tissue superfused with M199 *in vitro*. Samples were collected at 20 min intervals for 80 min, 5 min intervals for the next 25 min and 45 min intervals thereafter. (b) Mean amounts of steroids and prostaglandins in consecutive samples of superfusate from two chambers containing tissue superfused with M199 or cloprostenol in M199 (horizontal bar) *in vitro*. Samples were collected at 20 min intervals for 80 min, 5 min intervals for the next 25 min and 45 min intervals thereafter.

Tissues superfused with M199 alone throughout the experiment maintained secretion of progesterone, oestradiol-17$\beta$, prostaglandin $F_{2\alpha}$ and prostaglandin $E_2$ at an approximately constant level. The day 18 human luteal tissue was superfused *in vitro* for 21 h, and even after this time the amounts of steroids and prostaglandins being secreted were still 95–98% of that found for the early samples. The other tissues were maintained only for 8 h and amounts of steroids and prostaglandins in the last few samples were equivalent to those in the early samples. It appears therefore that the tissue was still viable after either 8 or 21 h superfusion with M199 *in vitro*.

Superfusion with cloprostenol (1 µg/ml) caused a depression of progesterone secretion in every case, but this did not persist. The greatest effect in terms of duration of depression of progesterone secretion was seen for the day 18 human luteal tissue sample. The depression of progesterone secretion from this tissue was followed by 4-fold increase of progesterone secretion which persisted after superfusion with cloprostenol had ceased. The other tissue samples did not show such a marked increase in progesterone secretions following the depression in secretion. The day 25 luteal tissue sample showed the smallest overall response to cloprostenol.

The pattern of oestradiol-17$\beta$ secretion following treatment with cloprostenol was similar for all tissues being an initial depression followed by a large potentiation. The greatest duration of depression of oestradiol-17$\beta$ secretion and subsequent potentiation was seen for the day 18 luteal tissue sample. The day 25 luteal tissue sample gave the smallest response to this treatment.

Superfusion with cloprostenol resulted in an increase in endogenous prostaglandin $F_{2\alpha}$ secretion, following a short lag phase, in every case. The day 18 luteal tissue sample gave the smallest response in relation to prostaglandin $F_{2\alpha}$ secretion, the increase being approximately 10-fold, while the other tissue samples (day 21 and day 25) showed a 15 to 20-fold increase in endogenous prostaglandin $F_{2\alpha}$ secretion.

The response of prostaglandin $E_2$ secretion to superfusion with cloprostenol was more variable but in every case an increase in secretion of endogenous prostaglandin $E_2$ was seen around the same time or just after the increase in prostaglandin $F_{2\alpha}$ secretion. However, the increase in prostaglandin $E_2$ secretion was not maintained, levels returning to control values before cessation of superfusion with cloprostenol.

## Discussion

Superfusion with cloprostenol (1 μg/ml) *in vitro* resulted in a reduction in progesterone secretion for all the human luteal tissue samples studied (days 18, 21 and 25 of the menstrual cycle). However the reduction in progesterone secretion was not maintained, the amounts of progesterone secreted returning to control or even higher values while superfusion with cloprostenol continued. Cloprostenol could not therefore be considered to be luteolytic for human luteal tissue *in vitro* at this dose (1 μg/ml) and under the conditions used for these experiments. The speed with which progesterone secretion was depressed (within 5 min for day 21 and day 25 luteal samples) could indicate an inhibition of release of progesterone.

A further, major, effect of the superfusion with cloprostenol was the large increase in secretion of endogenous prostaglandin $F_{2\alpha}$, which was seen in all samples, occurring after a lag phase of 15–45 minutes. A similar stimulation of endogenous prostaglandin $F_{2\alpha}$ secretion following treatment with cloprostenol has been observed in the sheep *in vivo* (Challis, Forster, Furr, Robinson & Thorburn, 1976b) but this followed a much longer lag phase (27–39 hours). The increased prostaglandin $F_{2\alpha}$ secretion seen for the human luteal tissue could be the result of progesterone withdrawal since inhibition of prostaglandin $F_{2\alpha}$ synthesis by progesterone has been demonstrated for human endometrial tissue *in vitro* (Cane & Villee, 1975). The appearance of endogenous prostaglandin $F_{2\alpha}$ in the uterine venous plasma of pregnant goats has also been observed following a decline in progesterone secretion resulting from treatment with exogenous prostaglandin $F_{2\alpha}$ *in vivo* (Currie & Thorburn, 1973) although the lag phase to increased endogenous prostaglandin $F_{2\alpha}$ secretion was again longer than observed in these experiments with human luteal tissue. In the guinea-pig (Poyser, 1976) and sheep (Barcikowski, Carlson, Wilson & McCracken 1974) oestradiol will initiate secretion of prostaglandin $F_{2\alpha}$ into the utero-ovarian vein and in woman intra-ovarian injections of oestradiol cause premature menstruation (Hoffman, 1960) suggesting a stimulation of luteolysis. However in these experiments with human luteal tissue *in vitro*, secretion of oestradiol-17β following cloprostenol treatment is depressed in every case until after the increase in secretion of endogenous prostaglandin $F_{2\alpha}$. It is hard to interpret the significance of either the initial depression or the subsequent stimulation of oestradiol-17β secretion seen for all the tissue samples in response to cloprostenol (1 μg/ml). Since the pattern of response for progesterone and oestradiol-17β are so similar it is tempting to consider that the changes in these steroids may be connected with each other. Although no direct comparison may be made between the *in vitro* and the *in vivo* situation, the possible effects of an increase in oestradiol-17β secretion at the level seen here could be an important consideration in relation to *in vivo* treatment with cloprostenol in humans.

The significance of the increase in prostaglandin $E_2$ secretion observed for all the human luteal tissue samples in response to cloprostenol is not clear.

The control (superfusion with M199 alone) levels of secretion of progesterone and oestradiol-17β agree broadly with those observed by Challis *et al.* (1976a) for static incubations. The levels of prostaglandins observed here for the control samples are, however, higher than those observed by Challis *et al.* (1976a). The prostaglandin $F_{2\alpha}$ levels were higher (100 ng $g^{-1}$ $h^{-1}$ and 70 ng $g^{-1}$ $h^{-1}$) than the prostaglandin $E_2$ levels (30 ng $g^{-1}$ $h^{-1}$ and 15 ng $g^{-1}$ $h^{-1}$) for the day 21 and day 25 human luteal tissue samples, respectively. The day 18 human luteal tissue sample, however, secreted more prostaglandin $E_2$ (125 ng $g^{-1}$ $h^{-1}$) than prostaglandin $F_{2\alpha}$ (16 ng $g^{-1}$ $h^{-1}$). These results contrast with those of Challis *et al.* (1976a) who found prostaglandin $E_2$ levels to be higher than prostaglandin $F_{2\alpha}$ levels in every case. However the conditions of our experiments differ extensively from those of Challis *et al.* (1976a) since we were using slices rather than homogenates and were also removing the secretory products of the tissue immediately, thus preventing any product-feedback which might otherwise occur. In this respect superfusion more closely resembles the physiological situation where many secretory products are continuously removed by the blood. Challis *et al.* (1976a) suggest that higher prostalandin levels may be indicative of the onset of cell autolysis. However,the maintenance of a constant pH (as gauged by medium colour) and maintenance of reasonable levels of steroid secretion throughout the experiments would tend to discount this possibility. In further experiments histological analysis of the tissue at the end of the experiments will answer this question.

The large increase in the secretion of endogenous prostaglandin $F_{2\alpha}$ by the human luteal tissue *in vitro* seen here in response to cloprostenol especially in day 21 and day 25 samples, indicates that the human corpus luteum has a very large potential for the secretion of prostaglandins. This observation supports the hypothesis of an intra-ovarian physiological rôle for prostaglandin $F_{2\alpha}$ in the human.

The authors recognize the limitations resulting from the small number of observations presented in this study but believe that the similarity of response obtained for each sample makes these observations worthwhile. Further work is in progress relating to cyclical variations in steroids and prostaglandins and the effects of prostaglandins on steroid and prostaglandin secretion by human luteal tissue superfused *in vitro*.

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# EFFECTS OF ATENOLOL ON REGIONAL MYOCARDIAL BLOOD FLOW AND ST SEGMENT ELEVATION IN THE CANINE MYOCARDIUM

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1 The effects of atenolol on regional myocardial blood flow (RMBF) and on ST segment elevation were studied both in normal and ischaemic regions of the myocardium in dogs. Some of the experiments were performed with cardiac pacing or after bilateral stellectomy.

2 In the absence of cardiac pacing, atenolol (1 mg/kg, i.v.) induced a marked reduction in heart rate and contractile force and a decrease in RMBF, which was of the same magnitude in normal and ischaemic areas. There was no modification in the endo/epi flow ratio. ST segment elevation in ischaemic areas was significantly reduced.

3 Bilateral stellectomy induced similar effects to those of atenolol. Atenolol after bilateral stellectomy exhibited no additional effects.

4 In dogs submitted to cardiac pacing, atenolol no longer decreased RMBF and ST segment elevation.

5 These results demonstrate that with atenolol, there is no correlation between bradycardia and the endo/epi flow ratio while there is one between bradycardia and reduction in ST segment elevation.

## Introduction

In recent years, several authors have shown, by assessing the degree and extent of ischaemic injury from epicardial electrocardiograms and/or creatinine phosphokinase release, that $\beta$-adrenoceptor blocking agents are capable of reducing 'infarct size' in dogs (Maroko, Kjekshus, Sobel, Watanabe, Covell, Ross & Braunwald, 1971) and in man (Pelides, Reid, Thomas & Shillingford, 1972). These beneficial effects of $\beta$-blockade are thought to be related to a more favourable balance between myocardial oxygen supply and demand in the ischaemic heart. However, other factors may contribute. For instance, Becker, Fortuin & Pitt (1971) and Gross & Winbury (1973) have shown that $\beta$-adrenoceptor antagonists can alter the distribution of regional myocardial blood flow (RMBF) and induce its redistribution from the epicardium to the endocardium in the normal and ischaemic heart. Moreover, Marshall & Parratt (1976) have recently shown in the canine myocardium that, while propranolol reduces RMBF in both ischaemic and non-ischaemic regions, practolol induces a more favourable distribution of coronary blood flow since it decreases RMBF only in the non-ischaemic areas; this beneficial effect has been partly attributed to the drug's $\beta_1$-selectivity.

In the present work we have investigated these two redistribution possibilities by studying the effects of atenolol, another selective $\beta_1$-adrenoceptor antagonist (Barrett, Carter, Fitzgerald, Hull & Le Count, 1973; Harry, Knapp & Linden, 1973) on RMBF assessed by the radioactive microsphere mapping technique (Becker, Ferreira & Thomas, 1973). This study has been performed in an experimental model of canine myocardial ischaemia (Berdeaux, Coutte, Giudicelli & Boissier, 1976a) with simultaneous measurement of ST segment changes. A preliminary account of this work has been published (Berdeaux, Boissier & Giudicelli, 1976).

## Methods

Twenty adult mongrel dogs of either sex, weighing 18–29 kg were anaesthetized with sodium pentobarbitone (35 mg/kg, i.v.). Artificial ventilation was performed by means of a Bird Mark 7 respirator using room air through a cuffed endotracheal tube. The chest was opened by means of a left thoractomy and the heart cradled in the open pericardium.

The first diagonal branch of the left anterior descending coronary artery was dissected free from the adjacent tissues and intermittently occluded with an intracranial arterial clamp. A strain gauge arch was sutured to the epicardial surface of the left ventricle in the area perfused by the circumflex coronary artery (non-ischaemic area) for the measurement of developed force. Silastic catheters were placed in the two femoral arteries and in the left atrial appendage. Blood pressure was measured with a Hewlett-Packard 267 AC transducer and displayed on a Hewlett-Packard 7700 multichannel recorder. The electrocardiogram (lead II) was monitored on a Visocardiette 560 Hewlett-Packard.

The experimental protocol was similar to that previously described (Berdeaux *et al.*, 1976a). In all dogs, a 12 min control occlusion was carried out with a microsphere injection 7 min after clamping and epicardial ST mapping just before and 2, 5 and 10 min after starting the occlusion. A second coronary artery occlusion was then performed usually 55 min later, i.e. at least 10 min after complete recovery from the first occlusion. This second occlusion, with the same ST mapping and blood flow measurements, gave similar results to the first (Berdeaux *et al.*, 1976a).

Four groups of five dogs each were studied. In group I, the animals were given atenolol (1 mg/kg, i.v.) 45 min after the first and 10 min before the second occlusion. In groups II and III, a bilateral stellectomy and a T1–T4 sympathetic chain removal was performed 45 min after the first and 30 min before the second occlusion. In addition in group III, atenolol (1mg/kg, i.v.) was given 10 min before the second occlusion. Group IV was identical to group I but atrial pacing (rectangular pulses, 2 V, 2 ms) at the pre-occlusion heart rate was started immediately before the atenolol injection and continued until the end of the second occlusion.

Myocardial blood flow was measured with radioactive tracer microspheres. The carbonized microspheres used were 15 ± 5 μm in diameter and labelled with the gamma emitting nuclides $^{141}$Ce or $^{85}$Sr (3 M Company). They were obtained as 1 mCi of nuclide suspended in 10 ml of 10% dextran with 1 drop of benzalkonium (0.5%). Aggregation of the microspheres before injection was prevented by vigorous stirring with a Teflon covered magnet and by applying ultrasound for 10 min (Ultrasonic model NSU 144). Approximately two million beads were injected into the left atrium for each blood flow determination. During each measurement a reference blood sample was collected from the femoral artery. Collection of the reference sample was started simultaneously with the beginning of microsphere injection and continued for 90 s at a rate of 20 ml/min, using a withdrawal pump (Sage Instruments, model 351).

At the end of the experiments, the animals were killed by an overdose of potassium chloride. The heart was then removed and fixed in buffered 4% formaldehyde for 2 days. Maps of the left ventricular myocardial blood flow were made according to the method of Becker *et al.* (1973). The free wall was cut into 40–50 pieces of approximately 0.5–1.5 g and the location of each piece of the free wall was recorded. All samples were divided into endocardial and epicardial halves, weighed and placed in vials for counting. The myocardial and blood samples were counted under similar geometry in a Nuclear Chicago Gamma Spectrometer at window settings that corresponded to the peak energies emitted by each radioactive nuclide (145 keV for $^{141}$Ce and 514 keV for $^{85}$Sr). The activities recorded in each energy window and the corresponding sample weights were then entered into a digital computer (Hewlett-Packard, Model 9821 A) programmed to correct activity recorded in each window for contaminant activity contributed by the associated nuclides and for background activity and to compute the corrected counts. Details of the design have been published by Schaper, Lewi, Flameng & Gipjen (1973). Knowing the rate of withdrawal of the reference sample ($\dot{Q}r$) and its radioactivity (Cr), we used myocardial activity (Cm) to compute myocardial blood flow ($\dot{Q}m$) as: $\dot{Q}m = \dot{Q}r \times Cm/Cr$. The flow for each sample was then calculated in ml min$^{-1}$ g$^{-1}$, maps of blood flow were drawn and endo/epi ratios were determined for each occlusion. According to Becker, Ferreira & Thomas (1975), and Berdeaux *et al.* (1976a), an 'ischaemic region' is defined as one in which flow is less than 50% of that of the non-ischaemic posterior wall. Pooled samples from this ischaemic region always had more than 400 spheres. Mean flows in endocardial and epicardial regions of the left ventricular wall and endo/epi ratios for each region were determined and compared before and after treatment, each dog serving as its own control.

Using the method of Maroko *et al.* (1971), tissue injury was assessed by ST segment elevation in epicardial electrocardiograms obtained from 12–15 sites on the anterior surface of the left ventricle. Some of the sites were within, and some outside, the ischaemic zone. Areas adjacent to vessel bifurcations were chosen for easy relocation. The exploring cotton wick epicardial electrode was soaked in saline and attached to lead V of a standard electrocardiograph. Elevation or depression of the ST segment was recorded at a speed of 25 mm/s (range 1 mV/mm) on the Hewlett-Packard 7700 recorder. Sites with ST elevation in excess of 2 mV before coronary occlusion were excluded from the study and mean ST segment elevation ($\overline{ST}$) was used as an index of the severity of ischaemia as described by Pelides *et al.* (1972).

The racemic form of atenolol hydrochloride was dissolved in 0.9% w/v NaCl solution (saline) and injected via a catheter in the saphenous vein. All doses refer to the salt. All values quoted in the text are means ± s.e. mean. Statistical analysis of the results was performed using Student's *t* test for paired data.

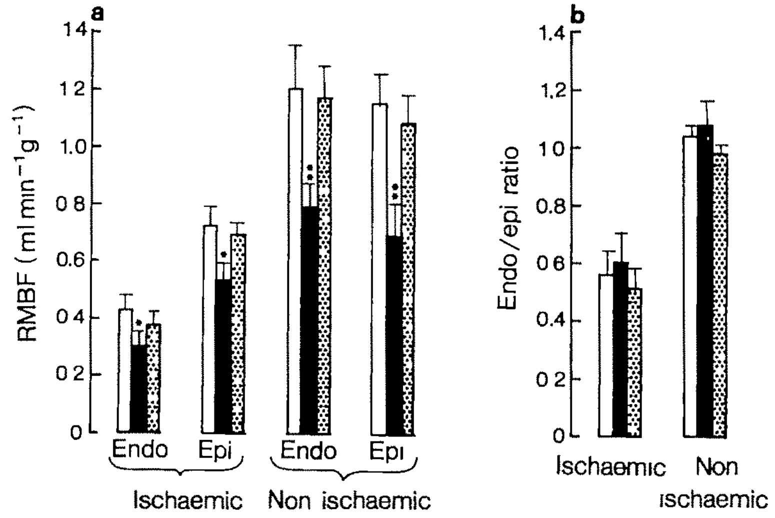

**Figure 1** Regional myocardial blood flow (RMBF) (a) in the endocardium and epicardium and (b) endo/epi ratio values measured in non-ischaemic and ischaemic areas of the canine myocardium, during a control coronary occlusion (open columns) and during occlusion performed after atenolol (1 mg/kg, i.v.) without (solid columns) and with atrial pacing (stippled columns). Values are means; vertical lines show s.e. mean. Significantly different from control occlusion values, *$P<0.05$; **$P<0.01$.

## Results

### *Group I* (Atenolol)

Table 1 shows the haemodynamic effects induced by atenolol, 1 mg/kg. There were significant decreases in heart rate and contractile force and a slight, but not significant, reduction in blood pressure.

Figure 1 compares the effects of the second occlusion, performed after atenolol, with those of the first occlusion performed before administration of the drug. RMBF values were significantly decreased and to the same extent both in ischaemic (–32.5 ± 6.2%) and non-ischaemic areas (–37.6 ± 7.5%). This decrease was similar in both epicardial and endocardial regions and this resulted in an unchanged endo/epi flow ratio in both ischaemic and non-ischaemic areas. Atenolol induced a significant reduction in the ST segment elevation during the second occlusion as compared to the first (Figure 2).

**Table 1** The effect of atenolol, bilateral stellectomy and atrial pacing on heart rate, contractile force and systemic arterial pressure

| | *Heart rate (beats/min)* | | *Contractile force (arbitrary units)* | | *Systolic blood pressure (mmHg)* | | *Diastolic blood pressure (mmHg)* | |
|---|---|---|---|---|---|---|---|---|
| *Treatment* | *Pre* | *Post* | *Pre* | *Post* | *Pre* | *Post* | *Pre* | *Post* |
| Atenolol (1 mg/kg)<br>$n=6$ | 174.1<br>±1.5 | 126.2‡<br>±3.1 | 14.3<br>±0.3 | 12.3*<br>±0.3 | 146.2<br>±5.5 | 142.5<br>±6.6 | 97.5<br>±2.5 | 96.5<br>±3.2 |
| Bilateral stellectomy<br>$n=5$ | 164.1<br>±1.7 | 110.0‡<br>±6.1 | 14.5<br>±0.8 | 10.6†<br>±0.4 | 161.2<br>±5.9 | 135.5†<br>±5.4 | 103.2<br>±5.2 | 88.7<br>±8.7 |
| Atenolol (1 mg/kg) after bilateral stellectomy $n=5$ | 154.1<br>±1.7 | 107.0‡<br>±7.2 | 14.5<br>±0.8 | 9.7†<br>±0.8 | 160.2<br>±5.2 | 133.7†<br>±8.6 | 100.2<br>±6.4 | 89.5<br>±8.8 |
| Atenolol (1 mg/kg) during atrial pacing $n=5$ | 153.0<br>±1.3 | 151.0<br>±7.1 | 16.0<br>±3.3 | 13.8<br>±3.6 | 149.0<br>±8.4 | 138.0<br>±6.3 | 86.0<br>±6.6 | 79.0<br>±6.1 |

Values are mean ± s.e. mean.
Significantly different from pre-treatment value *$P<0.05$; †$P<0.01$; ‡$P<0.001$.

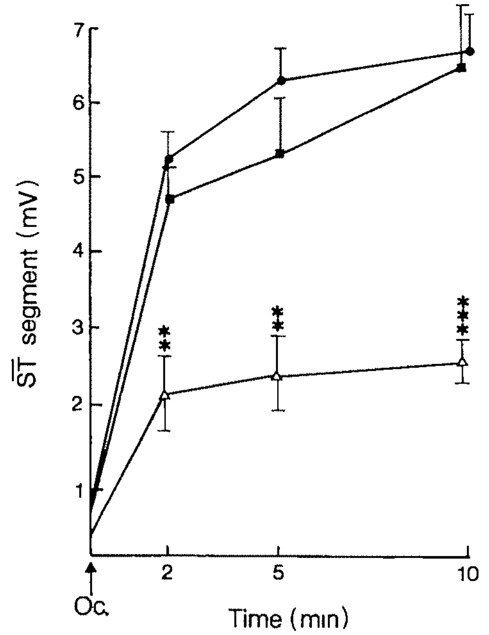

**Figure 2** Mean ST segment elevation ($\overline{ST}$) measured 2, 5 and 10 min after left anterior descending coronary artery ligation during control occlusion (●) and during occlusion performed after atenolol (1 mg/kg, i.v.) without (△) and with atrial pacing (■). Vertical lines show s.e. mean. Significantly different from control occlusion values, $^{**}P<0.01$; $^{***}P<0.001$.

*Group II* (Bilateral stellectomy)

Table 1 shows the haemodynamic effects induced by bilateral stellectomy. There were significant decreases in heart rate, contractile force and systolic blood pressure.

Figure 3 compares the effects of the second occlusion, performed after bilateral stellectomy, with those of the first occlusion performed before stellectomy. RMBF values were again significantly decreased, and to the same extent, both in ischaemic (−31.9 ± 6.4%) and non-ischaemic areas (−36.2 ± 8.7%). As in the group I dogs, this decrease was again similar in both epicardial and endocardial regions and the endo/epi flow ratio was thus not changed. After bilateral stellectomy, there was a significant reduction in the $\overline{ST}$ segment elevation during the second occlusion as compared to the first (Figure 4). This reduction was of a similar magnitude to that observed in the group I dogs.

*Group III* (Bilateral stellectomy and atenolol)

Table 1 summarizes the results obtained when atenolol was administered 10 min after bilateral stellectomy. Atenolol did not further reduce either heart rate or contractile force.

Figure 3 compares the effects of the second occlusion performed after bilateral stellectomy and atenolol to those of the first (control) occlusion. There were no differences between the effects observed in groups II and III: reduction in RMBF values was

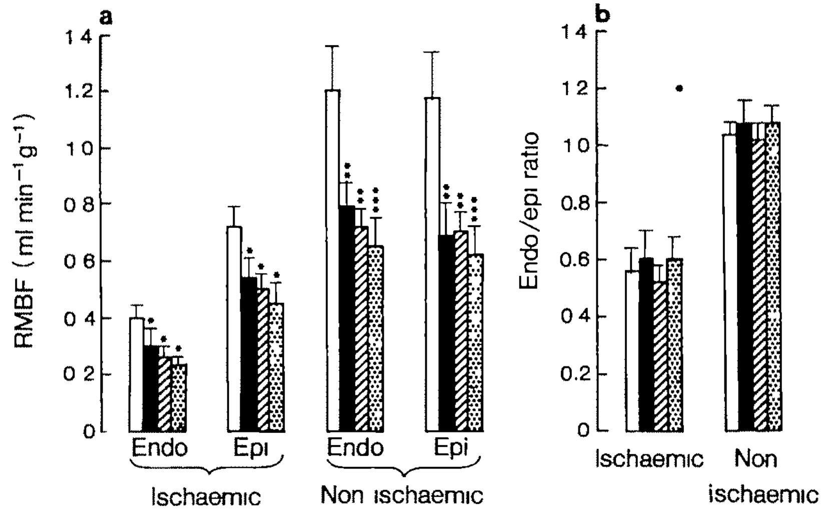

**Figure 3** Regional myocardial blood flow (RMBF) (a) in the endocardium and epicardium and (b) endo/epi ratio values measured in non-ischaemic and ischaemic areas of the canine myocardium, during a control coronary occlusion (open columns) and during occlusions performed either after bilateral stellectomy (hatched columns), after atenolol (1 mg/kg, i.v.) alone (solid columns), or after a combination of bilateral stellectomy and atenolol (stippled columns). Values are means; vertical lines show s.e. mean. Significantly different from control occlusion values, $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

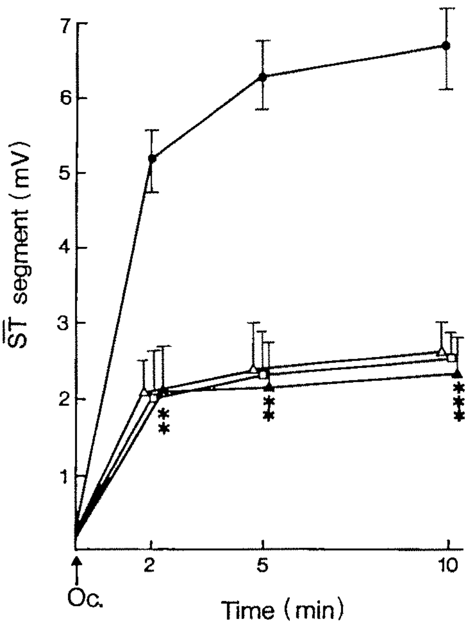

**Figure 4** Mean ST segment elevation ($\overline{ST}$) measured 2, 5 and 10 min after a control occlusion of the left anterior descending coronary artery (●) and during an occlusion performed after bilateral stellectomy (□), after atenolol (1 mg/kg, i.v.) (△) or after a combination of bilateral stellectomy and atenolol (▲). Significantly different from control occlusion values, $^{**}P<0.01$; $^{***}P<0.001$.

observed in both ischaemic and non-ischaemic areas and was homogeneous, i.e. endo/epi flow ratios remained unchanged. The reduction in $\overline{ST}$ segment elevation during the second occlusion as compared to the first was similar in groups III and II (Figure 4).

*Group IV* (Atrial pacing and atenolol)

Table 1 shows that the haemodynamic changes induced by atenolol during atrial pacing were not significant and almost negligible. During atrial pacing, atenolol no longer reduced RMBF either in the ischaemic or non-ischaemic areas and the endo/epi ratio remained unchanged (Figure 1). The reduction in $\overline{ST}$ segment elevation observed in group I was abolished when atenolol was given during atrial pacing (Figure 2).

## Discussion

Our results show that atenolol reduces regional myocardial blood flow both in ischaemic and non-ischaemic regions and that it does this without inducing any change in the endo/epicardial distribution of coronary flow. The reduction in regional flow is probably closely and directly related to the atenolol-induced decrease in myocardial oxygen consumption. It is logical then that the same phenomenon should be observed following bilateral stellectomy. This suggests that the decrease in contractile force, and particularly in heart rate, induced by atenolol and bilateral stellectomy is of greater relevance in reducing oxygen consumption than the drop in blood pressure observed after bilateral stellectomy alone. It is noteworthy that when atenolol-induced bradycardia was abolished by pacing, all changes in regional flow were suppressed. The fact that atenolol exerted no additional effects after bilateral stellectomy suggests that this drug reduces regional blood flow only by decreasing heart rate and contractile force (i.e. a reduction in oxygen consumption resulting from cardiac $\beta_1$-adrenoceptor blockade).

In non-ischaemic regions, adaptation of myocardial flow is accounted for by the phenomenon of metabolic autoregulation. In ischaemic regions, however, the reduction in local dilatory reserve impairs autoregulation capacity, and myocardial blood flow under these conditions is no longer dependent only on oxygen consumption (James, 1970). However, although at the centre of the lesion the capacity for dilatation is almost nil, numerous investigations have shown that the ischaemic region is in fact very heterogeneous with increasing flow gradients and autoregulation capacity from the centre to the periphery of the infarcting area (Becker *et al.*, 1973; Lubbe, Peisach, Pretorius, Bruyneel & Opie, 1974; Berdeaux *et al.*, 1976). The fact that our definition of the ischaemic region encompasses these various compartments accounts for the fact that we observed a reduction in regional flow under the influence of atenolol and bilateral stellectomy both in non-ischaemic regions and at the periphery of ischaemic areas. This reduction was of the same order of magnitude in these two regions. These results, which are consistent with those obtained with propranolol (Marshall & Parratt, 1976), contrast with the effects of practolol which does not reduce total regional blood flow in ischaemic regions. Marshall & Parratt (1976) have suggested two main reasons to account for this difference between propranolol and practolol. Firstly, only propranolol blocks coronary $\beta_2$-adrenoceptors (Parratt & Wadsworth, 1970; Gross & Feigl, 1975), and this alters the balance in favour of $\alpha$-vasoconstrictor receptors, hence reducing flow. Practolol, which does not block $\beta_2$-receptors, does not modify the $\alpha$–$\beta_2$ balance in the coronary arteries, thus leaving coronary flow unchanged. Secondly, practolol increases to a greater extent than propranolol, the diastolic effective perfusion period for the endocardium. Atenolol is a selective $\beta_1$-adrenoceptor inhibitor and therefore should not, like practolol, interfere with the $\alpha$–$\beta_2$ balance in the coronary arteries, and should thus leave myocardial flow unchanged. However, in practice, it reduces it. Two main reasons may explain this phenomenon. The first is that atenolol may lengthen to a lesser degree than practolol the diastolic effective perfusion period of the endocardium, a parameter we have not investigated. The second is that another factor is involved in the favourable effect of practolol, this factor possibly being its intrinsic $\beta$ stimulant effect (Dunlop & Shanks, 1968), a property of which atenolol and propranolol are devoid. This could exert a beneficial effect at the periphery of the ischaemic zone and thus limit the drop in myocardial flow.

In our experience, neither atenolol nor bilateral stellectomy redistributed blood flow between the epicardial and endocardial layers and the endo/epi ratio remained unchanged both in ischaemic and non-ischaemic regions. This distinguishes atenolol from propranolol since many investigators have reported a redistribution phenomenon with the latter drug (Becker *et al.*, 1971; Gross & Winbury, 1973; Becker *et al.*, 1975; Warltier, Gross & Hardman, 1976) although this has not been confirmed by other workers (Kloner, Reimer &Jennings, 1976). These results tend to demonstrate that there is no correlation between bradycardia and an increase in the coronary endo/epicardial flow ratio. Indeed, although bradycardia and redistribution are observed concomitantly with propranolol and other substances such as verapamil (Berdeaux, Coutte, Giudicelli & Boissier, 1976b), they are not seen after administration of atenolol or following bilateral stellectomy. In contrast, our experience has shown that there is a close relationship between bradycardia and reduction in ST segment elevation during coronary occlusion. Either atenolol or bilateral stellectomy, or a combination of both, simultaneously reduced heart rate and the degree of myocardial ischaemia. It would appear that this phenomenon may be applied more broadly to other substances that decrease heart rate, such as propranolol (Maroko *et al.*, 1971; Becker *et al.*, 1975), practolol (Libby, Maroko, Covell, Malloch, Ross & Braunwald, 1973; Marshall & Parratt, 1976) and verapamil (Berdeaux *et al.*, 1976b).

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# THE EFFECTS OF METIAMIDE ON CELL-MEDIATED IMMUNE REACTIONS IN THE GUINEA-PIG

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1 The effects of the $H_2$-receptor antagonist, metiamide, on two types of cell-mediated immune response *in vivo* was investigated in the guinea-pig, in a test of the hypothesis that $H_2$-receptor antagonists would result in 'runaway' unregulated hypersensitivity reactions.

2 Metiamide in doses of 5 mg–125 mg/kg given by 6-hourly injection during sensitization and challenge did not modify the delayed hypersensitivity reaction to tuberculin.

3 Metiamide in doses of 25–125 mg/kg given by injection, 6-hourly throughout sensitization to dinitrofluorobenzene (DNFB) and subsequent challenge six days later, did not modify the contact sensitivity reaction to DNFB.

4 It is concluded that metiamide, given during these cell-mediated immune reactions in the guinea-pig, does not enhance cell-mediated hypersensitivity responses.

## Introduction

It has recently been suggested that histamine, along with other endogenous pharmacologically active agents, may act *in vivo* to regulate the character and intensity of immunological and inflammatory responses (Bourne, Lichtenstein, Melmon, Henney, Weinstein & Shearer, 1974). Evidence has been put forward that, *in vitro*, histamine inhibits several processes involved in these responses such as direct lymphocyte-mediated cytotoxicity (Henney, Bourne & Lichtenstein, 1972), production of macrophage inhibitory factor (MIF) (Rocklin, 1976), lymphocyte activation (Rocklin, 1976; Beets & Dale, unpublished results), and histamine release from basophils (Bourne, Melmon & Lichtenstein, 1971; Lichtenstein & Gillespie, 1973). Rocklin (1976) has also claimed that histamine reduces delayed hypersensitivity reactions *in vivo*. The proposition, in general, is that histamine regulates immunological and inflammatory reactions, by acting on $H_2$-receptors on the cells concerned, resulting in an accumulation of cyclic adenosine 3,'5'-monophosphate (cyclic AMP), which inhibits or 'turns off' several cell functions. It is suggested that in doing so it serves 'to protect the host from the dangerous consequences of an unregulated immune response' (Bourne *et al.*, 1974).

If this is indeed so, one possible corollary could be that $H_2$-receptor antagonist drugs such as metiamide and cimetidine would exaggerate immunological and inflammatory reactions by preventing the suggested modulatory action of histamine. $H_2$-receptor antagonists are proving to be valuable in the treatment of peptic ulcer, Zollinger-Ellison syndrome, and other gastro-intestinal conditions (Thjodleifsson & Wormsley, 1975; Multicentre trial, 1975; Richardson & Walsh, 1976; MacDonald, Steele & Bottomley, 1976; Black, 1976). One of them, cimetidine, has recently been released for clinical use in this country and is likely to be widely prescribed. It is of considerable importance to know whether this group of drugs has a hidden potential for exacerbating any concurrent or intercurrent conditions with an inflammatory and/or anti-allergic component.

The present study attempts to determine whether such a hidden potential exists by examining the effect of a range of doses of metiamide on two animal models of cell-mediated immune reactions: the tuberculin reaction in the guinea-pig (the prototype of delayed hypersensitivity reactions) and contact sensitivity.

## Methods

### *Delayed hypersensitivity tests*

Eighteen female Hartley guinea-pigs were sensitized with complete Freund's adjuvant (CFA) and subsequently challenged with tuberculin (PPD).

*Sensitization.* Dried killed tubercle bacilli (10 mg) was mixed into 10 ml of complete Freund's adjuvant on a Whirlimixer. Each guinea-pig received 0.4 ml of this preparation injected into 3 sites: 0.2 ml intramuscularly into the back of the neck, 0.1 ml intradermally into the skin of the right ear and 0.1 ml intradermally into the skin of one hind foot.

*Challenge.* The skin was shaved on both flanks and 0.1 ml of tuberculin was injected intradermally, 5 μg on the left side and 50 μg on the right side.

*Drug treatment.* The 18 animals received the following subcutaneous injections in 1 ml/kg: Group I (5 animals) 0.9% w/v NaCl solution (saline); Group II (4 animals) metiamide 5 mg/kg; Group III (4 animals) metiamide 25 mg/kg; Group IV (5 animals) metiamide 125 mg/kg. The schedule of drug treatment was as follows: injections were started 24 h before sensitization and continued at 6-hourly intervals for 4 days subsequently. Two days before challenge the 6-hourly injections were started again and continued until the skin reactions had been read.

A nineteenth animal served as a 'blank' control, i.e. it was not injected with saline or metiamide and it was not sensitized with CFA but it was challenged with tuberculin at the same time as the others.

*Assessment of skin reactions.* At 24 h, erythema and skin thickness were measured in 'blind' conditions so as to avoid subjective biasing of the results. Two measurements of the diameter of the erythema were taken at right angles to each other with calipers, and the mean diameter calculated. Skin thickness was measured with Snelltaster skin calipers. Two measurements of normal skin thickness were taken on either side of the delayed hypersensitivity reaction, and the skin thickness measured at the actual site of the reaction was expressed as the % increase above the mean thickness of these two measurements.

### *Contact sensitivity tests*

Seventeen Hartley guinea-pigs were sensitized with dinitrofluorobenzene (DNFB) and subsequently challenged with DNFB 7 days later.

*Sensitization.* Fifty μl of 10% DNFB in a mixture consisting of equal parts of acetone and olive oil was applied to the right ear of each guinea-pig.

*Challenge.* The animals were shaved on both flanks and 4 areas marked out for treatment. Four different concentrations of DNFB were used: 0.05%, 0.1%, 0.25% and 0.5%, made up in a mixture of four parts of acetone to one part of olive oil. At each site 20 μl of the requisite solution was applied with an Eppendorf micropipette.

*Drug treatment.* The animals received the following injections: Group I (5 animals) saline; Group II (5 animals) metiamide 25 mg/kg; Group III (5 animals) metiamide 125 mg/kg. Injections were started 24 h before sensitization and continued at 6-hourly intervals for 5 days. On the fifth and sixth day they were given at 12-hourly intervals, then 6-hourly injections were recommenced and continued for a further 3 days.

Group IV (2 animals) received one injection of cyclophosphamide, 300 mg/kg intraperitoneally, three days before sensitization.

*Assessment of skin reactions.* The reactions to locally applied DNFB were rated on a 4-point scale (Turk, Parker & Poulter, 1972). All ratings were done 'blind'.

### *Drugs*

The following drugs were used: 1-fluoro-2,4-dinitrobenzene (Hopkin & Williams); cyclophosphamide (Koch-Light Laboratories); carboxymethyl-cellulose (sodium salt) (Sigma Chemical Co.); Freund's Complete Adjuvant (Difco Laboratories); metiamide (S.K. & F.); *M. tuberculosis*, heat killed strains C, DT and PN (Ministry of Agriculture, Fisheries and Food Central Veterinary Lab., Weybridge, Surrey).

## Results

### *Delayed hypersensitivity reactions to tuberculin*

The intensity of the delayed hypersensitivity reactions was assessed 24 h after challenge by measuring both the percentage increase in skin thickness and the diameter of the erythematous lesion at the site of the tuberculin injection. The results are given in Figure 1a (i) and (ii). The control guinea-pig showed no reaction at all at the injection site. All other animals showed a dose-related reaction to the injections of tuberculin, but there was no difference between the control group and the three groups treated with different doses of metiamide.

The dosage schedule chosen was based on preliminary experiments carried out by Caldwell (unpublished results). These showed that metiamide made up as a 10% solution in 0.5% carboxymethylcellulose and injected subcutaneously into the flank, gave fairly high blood levels for at least 6 h; for example, a dose of 250 mg/kg, produced the following mean blood levels (μM, with s.e. mean): at 2 h 224 ± 36; at 4 h 151 ± 17; at 6 h 69 ± 5; at 8 h 16 ± 6. Similar results were obtained when injections were made into the scruff of the neck.

In the tuberculin experiments of the present study, injections of metiamide or saline were started 24 h before sensitization and were carried on for 4 days i.e. during the initial critical phase of the immunological response to antigen. Injections were also given 6-hourly for 24 h before challenge and during the 24 h after challenge, up to the time when skin reactions were measured.

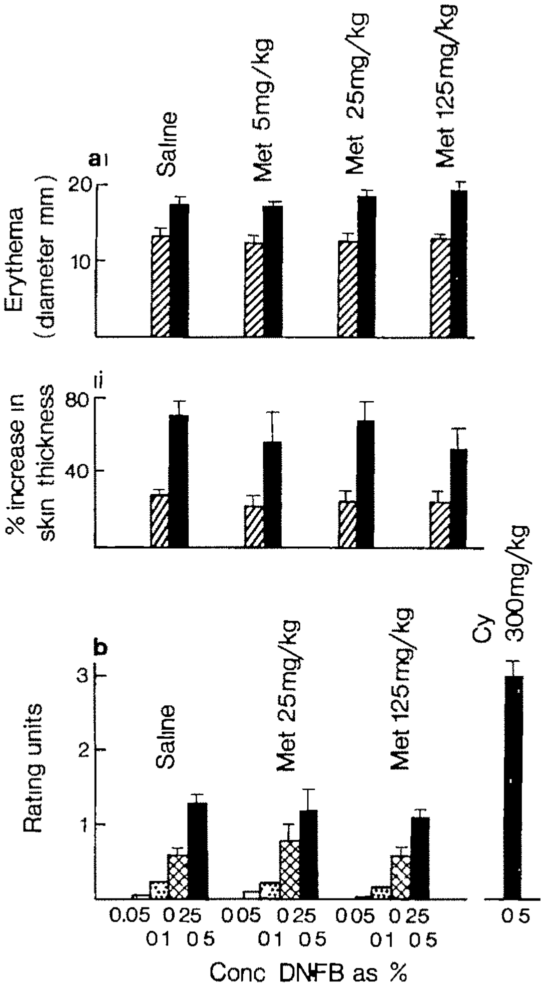

**Figure 1** Cell-mediated skin reactions in the guinea-pig (a) Skin reactions to two doses of tuberculin in 4 groups of guinea-pigs injected with saline or the indicated doses of metiamide (Met) as described in the methods section. Means are given; vertical lines show s.e. mean. All 4 groups had been sensitized to tubercle bacilli fourteen days before challenge with tuberculin. Measurements were made 24 h after challenge. Hatched columns, 5 µg tuberculin; solid columns, 50 µg tuberculin. (i) Diameter of erythema (mm). (ii) Induration of lesions given as % increase in skin thickness as compared with normal skin. (b) Contact sensitivity reactions to 4 doses of dinitrofluorobenzene (DNFB) in 4 groups of guinea-pigs injected with saline or metiamide (Met), or with one dose of cyclophosphamide (Cy) (see methods section). Means are given; vertical lines show s.e. mean. All groups had been sensitized with DNFB 7 days before challenge. The degree of reaction was rated on a 4-point scale 24 h after challenge.

Similar results were obtained when metiamide, 5 mg to 125 mg/kg was given at 6-hourly intervals during sensitization but not during challenge, or during challenge but not during sensitization.

### *Contact hypersensitivity reactions*

The skin reactions to DNFB were rated on a 4-point scale (Figure 1b). All animals showed a dose-related response to the challenging doses of DNFB. When the results were analysed by *t* tests, there was no significant difference ($P > 0.05$) between the reactions of the control group and those of the groups receiving metiamide. Metiamide had not therefore produced an increase in DNFB-induced contact hypersensitivity.

The lesions produced by challenge with a given concentration of DNFB were not the maximum possible reactions, i.e. the dose-response curve could be shifted to the left. This was demonstrated by the marked increase in the local reaction produced by pretreatment of the animals with cyclophosphamide (Turk *et al.,* 1972).

## Discussion

The tuberculin reaction is the prototype of cell-mediated delayed hypersensitivity reactions (Dvorak, 1974; Turk, 1967). The key cells in these reactions are considered to be the T-lymphocytes which in the presence of antigen transform and secrete a variety of mediators or lymphokines (such as MIF) which influence the activities of other cells including B-lymphocytes, macrophages, etc. (see review by Dvorak, 1974). The activated lymphocytes may also have direct cytotoxic effects. Both T and B-lymphocytes are purported to have or to develop on exposure to antigen, $H_2$-receptors (Henney *et al.,* 1972; Melmon, Bourne, Weinstein, Shearer, Bauminger & Kram, 1974), and thus to become susceptible to histamine which, produced locally during the reaction, is thought to modulate the response by a type of negative feedback (Bourne *et al.,* 1974). An $H_2$-receptor antagonist could thus be expected to prevent this negative feedback and lead to a runaway unregulated response. The presence of metiamide in the plasma during the critical phase of sensitization and challenge should therefore increase the tuberculin response in a dose-related fashion. In the present study there was no difference between the control animals and the metiamide-treated animals.

Contact sensitivity is another cell-mediated delayed hypersensitivity reaction, and the key cell is also believed to be a T-lymphocyte. Originally characterized as being cell-mediated by Landsteiner & Chase (1942), contact sensitivity is now included by some workers in the category of cutaneous basophil hypersensitivities (Dvorak, Simpson, Bast & Leskowitz, 1971; Dvorak & Dvorak, 1975), because one of the main phenomena is a local accumulation of basophils. In the guinea-pig, basophils may constitute 15 to 34% of the total infiltrating cells, which is all the more remarkable when it is considered that they are otherwise a relatively rare cell type, forming only 0.5% of circulating leucocytes. Basophils have a high content of histamine, which is released during immediate hypersensitivity reactions. Furthermore, basophils have been reported to have $H_2$-receptors which on stimulation modify histamine release (Lichtenstein & Gillespie, 1973). If this is the case, the effect would be altered by $H_2$-receptor antagonists. Metiamide should, in dose-related fashion, increase the intensity of the skin reactions. However, this was not found in the present study.

In the contact sensitivity experiments, a 'positive' control group was included, viz. guinea-pigs pretreated with cyclophosphamide, which is known to produce increased skin reactions. It has been suggested by Turk *et al.* (1972) that the cyclophosphamide depletes B-lymphocytes which would normally have a suppressive effect on the reaction. It is of interest that in animals pre-treated with cyclophosphamide no basophil infiltration in the contact sensitivity lesions was found (Katz, Heather, Parker & Turk, 1974).

It thus appears that metiamide does not exacerbate either tuberculin hypersensitivity or contact sensitivity in the guinea-pig even when it is continuously present at high concentration in the plasma. This does not necessarily mean that histamine is not an important regulator of these reactions, but its action probably involves factors other than negative feedback by stimulation of $H_2$-receptors.

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# C-FRAGMENT OF LIPOTROPIN— AN ENDOGENOUS POTENT ANALGESIC PEPTIDE

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**1** A series of peptides derived from porcine lipotropin was examined for analgesic and other morphine-like properties on infusion into the cannulated third ventricle of cats.

**2** Lipotropin (LPH 1-91) itself produced no analgesia or other morphine-like effects when infused in a dose of 150 µg.

**3** C-fragment (LPH 61-91) produced strong long-lasting analgesia when infused in a dose of 10 or 20 µg; on a molar basis the potency was between 90 and 180 times that of morphine. The following morphine-like effects were also produced: shivering leading to fever, vasodilatation of the pinnae, mydriasis, opening of the palpebral fissures, tachypnoea with bouts of panting, vocalization, hyperexcitability, restlessness and catalepsy. All the effects, including analgesia, were abolished by an intraperitoneal injection of naloxone (1 mg/kg).

**4** Hyperglycaemia, another central effect produced by morphine, was obtained with C-fragment infused in a dose of 60 µg.

**5** On intravenous injection, C-fragment produced analgesia with a dose of about 200 µg/kg. Administered by this route, C-fragment was again more potent than morphine.

**6** C′-fragment (LPH 61-87), LPH 61-78 and LPH 61-69, either had no analgesic effect or produced weak short-lasting analgesia when infused in doses up to 100 µg.

**7** Methionine enkephalin (LPH 61-65) either produced very weak short-lasting analgesia or had no analgesic effect when infused in doses of between 30 and 400 µg.

**8** *N*-methyl methionine enkephalin amide in which both termini of methionine enkephalin were protected against degradation by exopeptidases produced long-lasting analgesia when infused in doses of 150 to 180 µg; its analgesic potency was approximately 100 times less than that of C-fragment. Blocking only one terminus of methionine enkephalin did not appear to endow the peptide with analgesic properties. The *N*-methyl pentapeptide amide produced other morphine-like effects of which the most striking was catalepsy. All the effects were abolished by intraperitoneal naloxone (1 mg/kg).

## Introduction

The C-fragment of lipotropin, recently also called $\beta$-endorphin (Li & Chung, 1976), was shown to exist as an endogenous polypeptide when it was isolated from porcine pituitary (Bradbury, Smyth & Snell, 1975). This 31 residue peptide was found to have high affinity for the opiate receptors in brain homogenates (Bradbury, Smyth, Snell, Birdsall & Hulme, 1976). In the present experiments which have been communicated to the Physiological Society (Feldberg & Smyth, 1976, 1977) it was shown in cats that the C-fragment is a strong analgesic, much stronger than morphine, that it has other central morphine-like actions and that all the effects are abolished by the morphine antagonist, naloxone. On the other hand, the pentapeptide methionine enkephalin, which forms one end of the peptide chain of C-fragment and which interacts with the opiate receptors in the guinea-pig ileum, mouse vas deferens and brain homogenates (for references see Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975) had at most a very weak and short-lasting analgesic action.

In addition to C-fragment and methionine enkephalin, three related peptides intermediate in length were examined for analgesic and other morphine-like properties. So were three synthetic peptides obtained by blocking methionine enkephalin at one or both termini; this was done to render the peptide resistant to enzymatic destruction because the relative ineffectiveness of methionine enkephalin as an analgesic might be due to its rapid degradation *in vivo*, a suggestion made by Hambrook, Morgan, Rance & Smith (1976).

Since the first communication of our results, the strong analgesic action of C-fragment has also been obtained in rats (Graf, Szekely, Ronai, Dunai-Kovacs & Bajusz, 1976; Loh, Tseng, Wei & Li, 1976; Van Ree, de Wied, Bradbury, Hulme, Smyth & Snell, 1977; Bradbury, Smyth, Snell, Deakin & Wendlandt, 1977).

## Methods

Cats of either sex weighing between 2.7 and 3.7 kg were used. The peptides and morphine were infused into the third ventricle caudal to the massa intermedia in a volume of 40 µl by a microinfusion pump which delivered this volume from a 100 µl syringe in 4 min 20 seconds. The infusions were made through a Collison cannula implanted into this part of the third ventricle in a preliminary aseptic operation under pentobarbitone sodium anaesthesia (36 mg/kg, i.p.) as described previously (Feldberg & Shaligram, 1972). After recovery from the operation the infusions were made without anaesthesia, the stilette being removed from the Collison cannula and a hollow stainless-steel needle (28 gauge) inserted instead. The length was such that when the needle passed through the rubber diaphragm of the cap and was fully inserted, it ended just beyond the end of the shaft of the cannula. The needle was connected by a length of fine Polythene tubing to the syringe of the microinfusion pump, needle and tubing being filled with the solution to be infused and the syringe with absolute alcohol.

### *Analgesia*

To test for analgesia, the tail-pinch method of Russell & Tate (1975) was used in which a small diameter rod presses across the root of the tail. Graded pressure is applied to the tail for a 5 s period at about 1 min intervals. The pressure is exerted hydraulically and measured with a Bourdon gauge in kg/cm$^2$. Although this pressure is not identical with the force exerted on the tail it is proportional to it and is given without conversion as ordinates in the figures. The responses obtained with increasing pressure were classified as none, weak, medium or strong, according to the description given by Dey & Feldberg (1976). A weak response indicated that the cat noticed the pressure, but whether it felt pain was not certain. A medium response indicated definite pain and a strong response indicated intense pain. In the figures the signs 'none' and 'weak' are supplied with an interrupted vertical line. The length of the lines indicates the pressures which can apparently be exerted on the tail without eliciting pain and the increase in the height of the lines, the degree of analgesia obtained.

### *Catalepsy*

To test for signs of catalepsy the cat was put in a nearly erect position with its forepaws placed over the upper rung of an inverted stool, or the cat was placed across two upper rungs of the stool. If cataleptic, the cat would retain these postures for several seconds or even for a few minutes without struggling, but its movements were not impaired. When climbing down the cat moved away, and when gently pushed from behind it jumped in a well co-ordinated manner. These simple procedures have previously been used to test for catalepsy produced in rabbits and cats by various drugs (Feldberg & Sherwood, 1954; 1955) including morphine (Banerjee, Burks, Feldberg & Goodrich, 1968; Feldberg & Shaligram, 1972; Dey & Feldberg, 1976).

### *Blood glucose*

In one experiment blood glucose estimations were made. About 1.5 h before the first blood sample was withdrawn a nylon catheter was inserted, during a short-lasting Althesin anaesthesia, about 15 mm deep into the right femoral vein so that the opening was lying in the inferior vena cava, caudal to the entrance of the hepatic veins. The outer end of the catheter, closed by a rubber cap, was taken over to the outside of the thigh and held in position with adhesive tape. The patency of the catheter was ensured by flushing it with an injection of 1 ml of 0.9% w/v NaCl solution (saline) containing 50 u heparin through the cap after each collection of a blood sample. Further, to prevent the blood samples from being contaminated with the saline-heparin solution or blood stagnating in the catheter, 0.75 ml of blood was withdrawn immediately before a blood sample was taken, and discarded. To prevent coagulation, the blood samples (2 ml) were collected in ammonium fluoride and ammonium oxalate as described by Anderson (1969). Glucose concentration was determined with the automated glucose oxidase method described by the Sigma Technical Bulletin No. 970 (1970).

### *Peptides and drugs*

For lipotropin (LPH) and the peptides which form part of its peptide chain the number of amino acids and their position in the chain are given together with the molecular weight (mol. wt.). Porcine lipotropin (LPH 1–91) which has a mol. wt. of 9894 was isolated from porcine pituitary gland according to the method of Li, Barnafi, Chretien & Chung (1965) as modified by Bradbury *et al.* (1975). C-fragment (LPH 61–91, mol. wt. 3423.7) and C′-fragment (LPH 61–87, mol. wt. 2982.3) were isolated by extraction of porcine pituitary glands and obtained in homogeneous form by ion exchange chromatography (Bradbury, Smyth & Snell, 1975; 1976). About 30 mg of C-fragment and 10 mg of C′-fragment were obtained from 1200 pituitary glands. The octadecylpeptide LPH 61–78 (mol. wt. 2006.1) was obtained by mild chymotrypsin digestion of C-fragment (1:500) for 1 h at 37°C; its isolation was by chromatography on a column (40 x 1 cm) of SP25 with a gradient from 0–1M sodium chloride as eluent at pH 7.0 (Massey & Smyth, unpublished experiments). The nonapeptide LPH 61–69 (mol. wt. 1019.2) and the pentapeptide methionine enkephalin (LPH 61–65, mol. wt. 573.7) were synthesized by Bradbury & Smyth (unpublished experiments) using the solid phase method of Merrifield (1963). *N*-methyl methionine enkephalin (mol. wt. 587.8), methionine enkephalin amide (mol. wt. 572.8) and *N*-methyl methionine enkephalin amide (mol. wt. 586.8) were prepared by Bradbury, Smyth & Snell (unpublished experiments). The morphine preparation used was morphine sulphate B.P. (MacFarlane Smith Ltd, Edinburgh). Its mol. wt. is 758.8 but the salt contains two molecules of morphine. Therefore, for comparing its activity with those of the peptides on a molar basis, half the mol. wt. was taken. Naloxone hydrochloride (Endo Laboratories, New York) was used.

## Results

### *Lipotropin (LPH 1–91)*

Infused into the third ventricle in a dose of 150 µg lipotropin produced no analgesia or other morphine-like effects.

### *C-fragment (LPH 61–91)*

Infused into the third ventricle in a dose of 10 or 20 µg C-fragment produced strong long-lasting analgesia. In cats in which the effect was compared with that of morphine, analgesia was as strong though not as long lasting as that produced by 100 or 200 µg of morphine sulphate similarly infused. On a molar basis the analgesic potency of C-fragment was between 90 and 180 times that of morphine.

The analgesia produced by 10 µg of C-fragment is illustrated in Figure 1a. It developed within a few minutes, reached its maximum in about 30 min and after about 1.5 h began to subside slowly. Some analgesia was still present 1 h later, i.e. about 2.5 h after the infusion. In this cat the analgesia which developed following the infusion of 200 µg of morphine sulphate was stronger and longer-lasting than that following the infusion of 10 µg of C-fragment. This is shown in Figure 1b which is a record obtained from the same cat nine days later. No analgesia developed on infusion of 2.5 µg of C-fragment.

On intravenous administration through the cephalic vein of a foreleg, C-fragment was less potent; yet it was again more potent than morphine. In the experiment of Figure 2, 0.57 mg of C-fragment injected intravenously into a 3 kg cat produced definite analgesia lasting about 20 min, whereas 1 mg of morphine sulphate similarly injected had no analgesic effect. In another cat weighing 2.5 kg, an intravenous injection of 0.62 mg of C-fragment produced analgesia of approximately the same intensity lasting about 30 minutes.

The infusion into the third ventricle of 10 or 20 µg of C-fragment produced other effects which are obtained when morphine is introduced into the liquor space (Feldberg & Shaligram, 1972) such as shivering resulting in fever, vasodilatation in the pinnae, tachypnoea with bouts of panting, mydriasis, widening of the palpebral fissures, vocalisation, hyperexcitability with restlessness and signs of catalepsy. Of these effects, shivering, vasodilatation of the pinnae and tachypnoea with panting occurred also when C-fragment was infused in a dose (2.5 µg) sub-threshold for analgesia.

Shivering was the first effect to appear. It began during the infusion of C-fragment, quickly became vigorous and continued for periods varying between 20 and 90 minutes. Figure 3 shows the rise in rectal temperature resulting from such shivering following the infusion of 10 µg of C-fragment.

Vasodilatation of the pinnae and tachypnoea began a few minutes after the infusion. Vasodilatation disappeared after 20 to 90 minutes. Tachypnoea developed rapidly into bouts of panting which continued for up to 2 hours. The time course for mydriasis, opening of the palpebral fissures and vocalization was about the same. The pupils became maximally dilated and the eyes opened wide. In this condition the cats with their ears pricked would stare vacantly without blinking for long periods. Vocalization consisted of bouts of miaowing which occurred at shorter and shorter intervals and with increasing duration until it became continuous, the miaowing becoming louder and more frequent.

Hyperexcitability and restlessness developed later than the other effects, occurring 30 min or more after the infusion. During the state of hyperexcitability the cat, when lightly touched, made rapid, brisk movements usually leading to circling in the relatively small cage. Later on, the movements leading to circling occurred spontaneously. Signs of catalepsy were observed in a few experiments, 1–1.5 h after the infusion. When placed in a nearly erect position with their forepaws over the upper rung of an inverted stool, the cats retained the posture for 30 s or longer.

C-fragment in large doses, like morphine, was found to be strongly hyperglycaemic, as illustrated by the experiment of Figure 4. The blood glucose concentration rose from 60.0 and 63.5 before, to 280 mg/100 ml 1.5 h after the infusion of 60 µg of C-fragment, whilst strong analgesia developed. When tested after the fourth and fifth blood sample the cat did not react at all to the maximum pressure of 4 kg/cm$^2$ exerted on

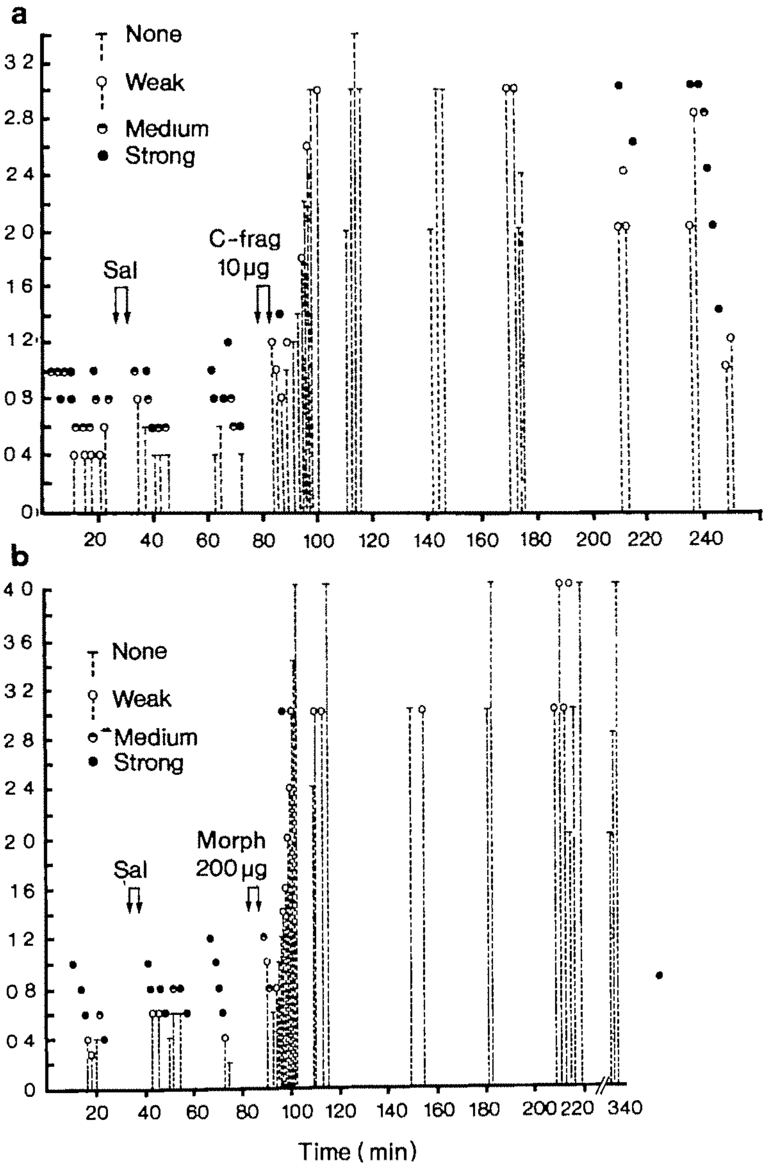

**Figure 1** Analgesia produced in a cat (3.2 kg) (a) on infusion of 10 µg of C-fragment (C-Frag) and (b) of 200 µg of morphine sulphate (Morph) into the third ventricle; (b) was obtained 9 days after (a). Sal = control infusion of 40 µl saline In this figure as well as in Figures 2, 5, 6 and 7, the ordinates give the pressure in kg/cm$^2$ exerted for 5 s on the root of the tail, and the degree of analgesia is indicated by the rise in the interrupted lines.

the tail. In Figure 4 the hyperglycaemic effect is compared with that previously obtained (Feldberg & Shaligram, 1972) with 750 µg of morphine sulphate injected into a lateral ventricle. The three curves give the mean, the maximal and the minimal results obtained in nine cats.

### *Intermediate LPH fragments*

LPH 61–87, 61–78 and 61–69, either had no analgesic effect or produced short-lasting weak analgesia when infused in doses up to 100 µg. Reducing the peptide chain of C-fragment by only

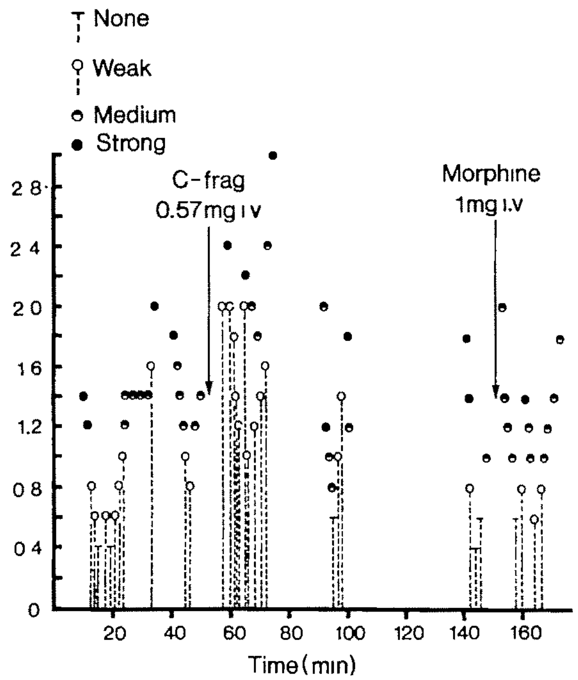

**Figure 2** Analgesia produced in a cat (3 kg) on intravenous injection of 0.57 mg of C-fragment (C-Frag). Comparison with intravenous injection of 1 mg of morphine sulphate.

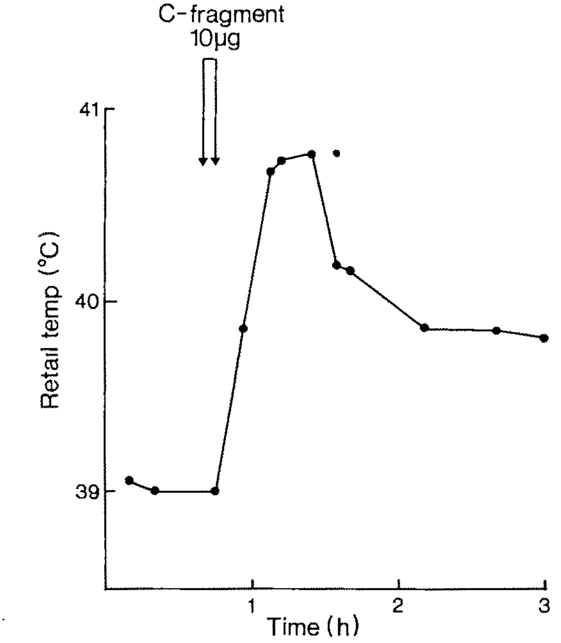

**Figure 3** Fever produced in a cat (3.0 kg) on infusion of 10 µg of C-fragment into the third ventricle. Ordinates: rectal temperature in °C.

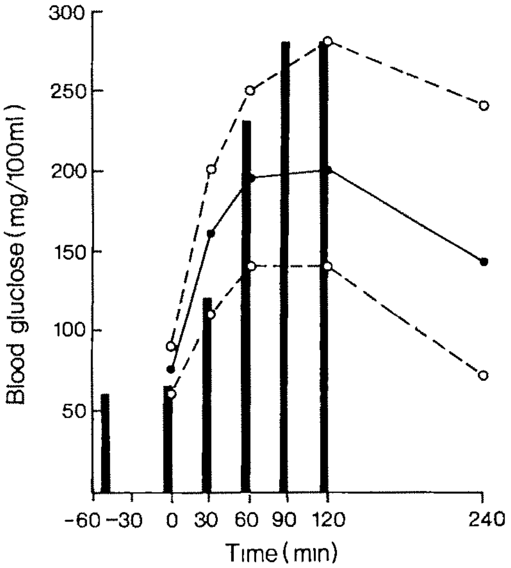

**Figure 4** Hyperglycaemia produced in a cat (3.0 kg) on infusion of 60 µg of C-fragment into the third ventricle. Ordinates are mg glucose in 100 ml blood. The vertical columns give the times when venous blood samples were taken before and after the infusion and the height of the columns their glucose concentration. The three curves are plotted from experiments of Feldberg & Shaligram (1972) and give for comparison the maximal, mean and minimal hyperglycaemic effects produced in nine cats on infusion of 750 µg of morphine sulphate into a lateral ventricle

four amino acids resulted in a loss of its analgesic property because the C′-fragment (LPH 61–87) infused in doses of 30 to 100 µg produced no analgesia or other morphine-like effects. Larger doses were not tested.

LPH 61–78 was infused in one cat first in a dose of 50 and later of 100 µg. Both infusions produced some shivering and the infusion of the larger dose was followed by weak analgesia which lasted less than 20 min. The infusion of the nonapeptide LPH 61–69 in a dose of 100 µg had no analgesic effect, but it produced some shivering and vasodilatation in the pinnae.

### *Methionine enkephalin (LPH 61–65)*

The pentapeptide had at most a very weak and short-lasting analgesic effect when infused in doses of between 30 and 200 µg. In some of the experiments the pressure that could be exerted on the tail without producing pain responses was a little stronger during the first few minutes following the infusion than before it, suggesting a weak short-lasting analgesia. However,

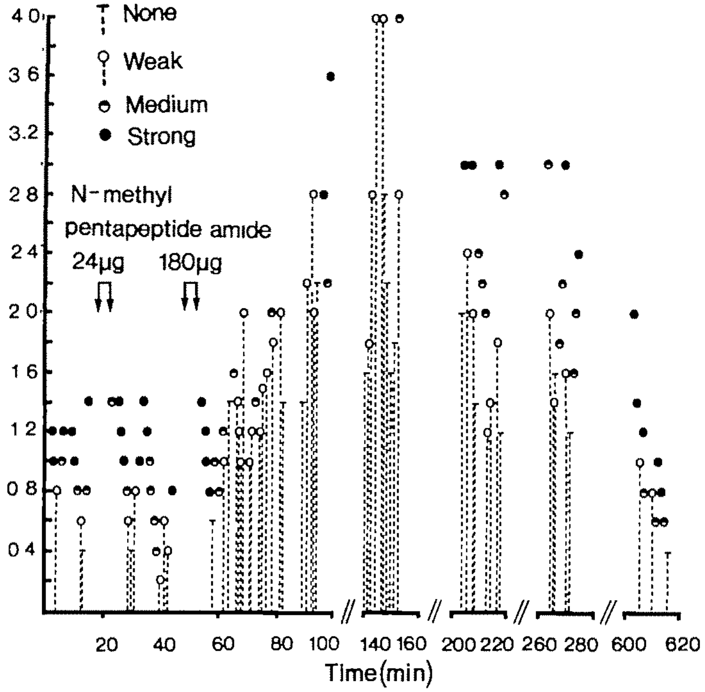

**Figure 5** Analgesia produced in a cat (2 8 kg) on infusion of 180 µg *N*-methyl methionine enkephalin amide into the third ventricle. No analgesia on infusion of 24 µg.

the effect was not dose-dependent; in one experiment it did not occur on infusion of 400 µg. On the other hand weak, short-lasting analgesia was sometimes obtained following control infusions of saline. With the larger doses of methionine enkephalin some shivering, tachypnoea and in one experiment panting were obtained.

### N-*methyl methionine enkephalin amide*

The pentapeptide amide produced strong analgesia when infused into the third ventricle in doses of 150 to 180 µg. It was less potent than C-fragment since it was ineffective in doses of 10 to 30 µg. The effect of 24 and 180 µg is shown in Figure 5. After 24 µg no analgesia developed; after 180 µg analgesia developed within a few minutes, gradually increased and became maximal in about 1.5 hours. It then gradually diminished but had not fully subsided 3.5 h after the infusion. On a molar basis the analgesic potency of the *N*-methyl methionine enkephalin would appear to be about one hundredth that of C-fragment since the effect of 150 to 180 µg corresponded to that of 10 µg of C-fragment.

The infusion of 150 to 180 µg of the *N*-methyl methionine enkephalin amide produced in addition to analgesia, shivering and vasodilatation of the ear vessels as well as some mydriasis and tachypnoea, but less pronounced than following the infusion of C-fragment. Mydriasis did not become maximal and the rate of respiration did not exceed 80/minute. The most striking effect, apart from analgesia was the deep stupor and catalepsy which developed within 15 min of the infusion, became maximal during the first hour and then gradually subsided during the next hour although analgesia continued for some time. During the condition of deep catalepsy the cat tended to lie on its side and made no effort to get up when touched or handled. When put into a nearly erect position with its forepaws over the upper rung of an inverted stool, or when placed horizontally across the two rungs, the cat did not resist and retained these postures for a few minutes; but when it subsequently moved away it showed no signs of ataxia. The condition of catalepsy was followed by one in which the cat became extremely affectionate, a condition also seen after morphine infusion. Finally about 4 h after the infusion the cat became very sleepy.

Both ends of the methionine enkephalin had to be blocked for the pentapeptide to become an analgesic substance. Blocking the C-terminus with an amide group did not endow the pentapeptide with analgesic or other morphine-like properties. Infused in a dose of 200 µg, methionine enkephalin amide produced no

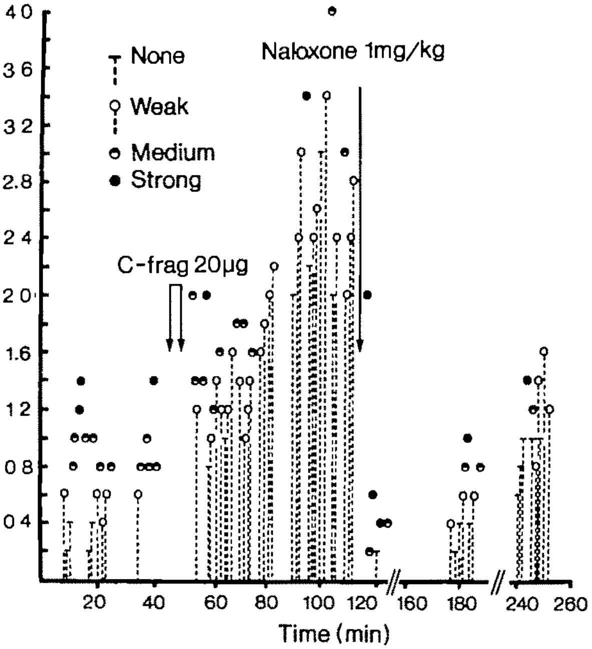

**Figure 6** Analgesia produced in a cat (2.8 kg) on infusion of 20 μg of C-fragment (C-frag) into the third ventricle, reversal by naloxone (1 mg/kg i.p.).

analgesia; the only effect observed was some shivering. Blocking the other end of methionine enkephalin with an *N*-methyl group produced a pentapeptide of relatively low solubility which could be infused only in a dose of 70 μg. This dose produced no analgesia, shivering or other morphine-like effect.

### *Naloxone*

Analgesia as well as the other morphine-like effects produced on infusion of morphine sulphate, C-fragment and *N*-methyl methionine enkephalin amide were abolished within a few minutes of an intraperitoneal injection of naloxone 1 mg/kg.

The effect of naloxone on the analgesia produced by the infusion of 20 μg of C-fragment is illustrated in Figure 6. Within 3 min of the naloxone injection, analgesia had disappeared. In this experiment the cat became even more sensitive to the pressure exerted on the tail during the first 10 min following the naloxone injection. The experiment illustrates another feature, the return of the analgesic effect as the naloxone effect subsided. Before the naloxone injection the pupils were dilated, the cat was stuporous, staring with wide open eyes, rarely blinking, and there were definite signs of catalepsy. Within the first 3 min of the injection, mydriasis began to disappear, the eyes were no longer wide open, blinking occurred frequently and the cat became lively and alert. Later the pupils became slit-like and all signs of catalepsy were found to have disappeared.

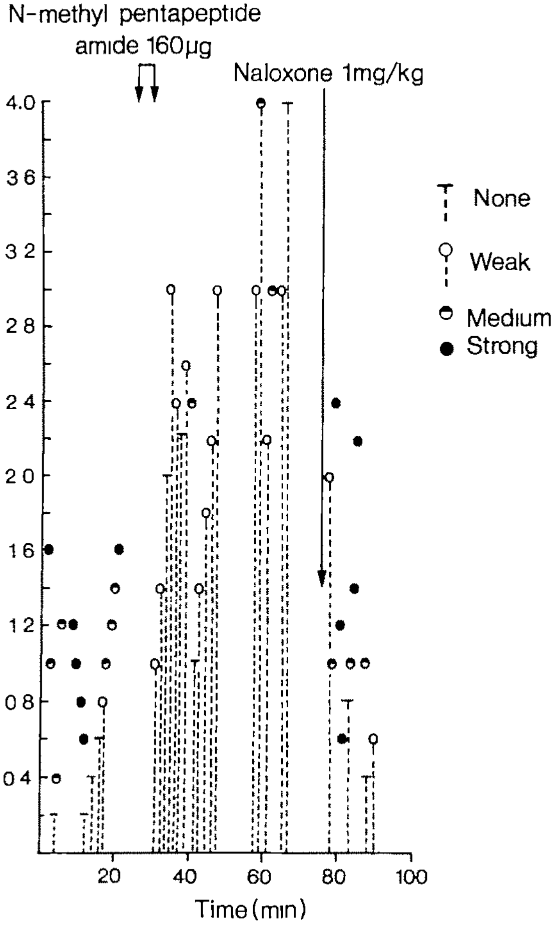

**Figure 7** Analgesia produced in a cat (2.7 kg) on infusion of 160 μg of *N*-methyl methionine enkephalin amide into the third ventricle; reversal by naloxone (1 mg/kg i.p.).

The effect of naloxone on the analgesia produced by the infusion of 160 μg of the *N*-methyl methionine enkephalin amide is illustrated in Figure 7. Again analgesia disappeared within the first three minutes. Before the naloxone injection the cat was shivering, stuporous, lying on its side and deeply cataleptic. It was striking that within 2 min of the injection, shivering stopped, stupor disappeared, the cat got up, became lively and alert and when tested 4 min after the injection, no longer showed any signs of catalepsy.

## Discussion

The main result of the present experiments is the strong analgesia produced by C-fragment and the reversal of this effect by the morphine antagonist naloxone. The analgesic action of C-fragment was found to be stronger than that of morphine whether administered by infusion into the third ventricle or injection into the cephalic vein. On infusion the potency of C-fragment was between 90 and 180 times greater than that of morphine. A closer comparison was not attempted because relatively small amounts of C-fragment were available and the number of experiments was limited.

Although the main clinical use of morphine is based on its analgesic property, morphine produces other central effects which vary in different species. Those produced in cats were also obtained with C-fragment; one of them was hyperglycaemia. For this effect larger doses both of morphine and of C-fragment are required than those necessary to produce analgesia. As a hyperglycaemic agent, too, C-fragment appeared to be at least 100 times more potent than morphine, though it has to be pointed out that the methods used for comparing the potency of the two substances were slightly different, C-fragment being infused into the third ventricle whereas morphine was injected into a lateral ventricle.

Recently it was shown (Belluzi, Grant, Garsky, Sarantakis, Wise & Stein, 1976) that the injection of methionine enkephalin into the cerebral ventricles of rats results in a short-lasting weak analgesia. In our experiments with infusion into the third ventricle of cats, no definite evidence for such an action was obtained. It may be that with our relatively slow method of infusion, a weak short-lasting analgesic effect would not be revealed. Alternatively methionine enkephalin might simply be inactivated more rapidly by the peptidases in the cat than in the rat.

If the ineffectiveness of methionine enkephalin as an analgesic is due to its rapid destruction by exopeptidases *in vivo* as suggested by Hambrook *et al.* (1976), making it more resistant to these enzymes should produce an analgesic peptide. To achieve this the two termini of the pentapeptide were blocked, one by an *N*-methyl, the other by a *C*-amide group. The resulting *N*-methyl pentapeptide amide was found to produce long-lasting analgesia when infused into the third ventricle; but its potency as an analgesic was less than one hundredth that of C-fragment. This result resembles that obtained by Pert, Pert, Chang & Fong (1976) who substituted glycine at position 2 of methionine enkephalin by the D-isomer of alanine and found that the modified enkephalin gave long-lasting analgesia on injection into the cerebral ventricles of rats. Again it was less potent than morphine and therefore much less potent than C-fragment. The *N*-methyl enkephalin amide also produced other morphine-like effects. Catalepsy was particularly striking and was more pronounced than that obtained with C-fragment, suggesting that the relative potencies of different morphine-like effects may vary with different analgesic peptides.

Methionine enkephalin which forms one end of the peptide chain of C-fragment, cannot be solely responsible for its strong analgesic action because the C′-fragment which lacks only four amino acids at the C-terminus exerted no analgesic or other morphine-like effect when infused in doses much greater than those effective with C-fragment.

When considering the possible physiological roles of C-fragment and methionine enkephalin in reducing pain sensation in the body, the fact has to be taken into account that C-fragment is not only a much stronger analgesic than methionine enkephalin but is by far the most potent analgesic peptide known to occur endogenously. It is also a stronger analgesic than any of the synthetic pentapeptides obtained by modifying the enkephalin molecule so as to render it less liable to enzymatic destruction *in vivo*. There are in principle three possibilities of how a naturally occurring analgesic peptide might exert its analgesic function.

First, it may reach the nervous structures in the brain via the blood stream by being released into the circulation. This possibility can probably be excluded for methionine enkephalin because of its rapid destruction by enzymes in blood (Hambrook *et al.* 1976); but it has to be considered for C-fragment. Not only is C-fragment much more stable than methionine enkephalin to degradation by enzymes present in brain homogenates (Austen & Smyth, unpublished experiments) but it is also effective on intravenous injection. Although about 30 to 60 times larger doses were required than on infusion into the third ventricle, this is the usual difference in efficacy with these two routes of administration for drugs acting on structures lining the ventricular walls or the ventral surfaces of the brain stem.

Second, the peptide may reach the nervous structures via the liquor space by being released into the cerebral ventricles or into the subarachnoid space. For methionine enkephalin this possibility can probably also be excluded because of its relative ineffectiveness in producing analgesia when infused into the third ventricle. On the other hand, C-fragment produces strong analgesia when applied by the intraventricular route. If C-fragment were to be released from the pituitary into the liquor space one would expect it to be released not into the cerebral ventricles but into the subarachnoid space beneath the ventral surface of the brain stem and it would then have to exert its analgesic effect from this site. This should create no difficulty. Morphine for instance, infused into the subarachnoid space beneath the ventral surface of the brain stem produces analgesia. In fact, its analgesic effect on injection into the cerebral ventricles is thought to be due, at least in part, to an action on structures at the ventral surface of the brain stem after having passed from the ventricles into the subarachnoid space (Dey & Feldberg, 1976).

Third, the analgesic peptides could be released during neuronal activity perhaps as transmitter substances from nerve endings and act locally at the site of release. This has been considered as a mechanism by which methionine enkephalin might produce its analgesic effect (Smith, Hughes, Kosterlitz & Sosa, 1976). Its rapid destruction *in vivo* might in fact be an advantage for such a transmitter function, but there is no experimental evidence for a transmitter function as yet. Such a mechanism could not be envisaged for the C-fragment released from the pituitary but the C-fragment occurs also in brain tissue (Bradbury, Feldberg, Smyth & Snell, 1976). For the extra-pituitary C-fragment a transmitter function can be envisaged even though the known transmitters are relatively small molecules. As a transmitter for its analgesic and other morphine-like effects the action of C-fragment would not necessarily be evanescent.

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# MECHANISMS UNDERLYING GASTRIC MUCOSAL DAMAGE INDUCED BY INDOMETHACIN AND BILE-SALTS, AND THE ACTIONS OF PROSTAGLANDINS

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1 The mechanisms by which the bile salt, sodium taurocholate, potentiates the formation of gastric mucosal erosions induced by indomethacin has been investigated in the rat.

2 Systemic administration of indomethacin lowered resting mucosal blood flow but had no effect on the acid back-diffusion across the mucosa.

3 Gastric perfusion of taurocholate in acid solution increased acid back-diffusion and lowered the potential difference. This was accompanied by an increase in mucosal blood flow, which may represent a protective mechanism in the mucosa.

4 Administration of indomethacin during acid-taurocholate perfusion reduced this elevated mucosal blood flow without any further change in acid back-diffusion.

5 The results suggest that although a decrease in mucosal blood flow or an increase in acid back-diffusion can lead to a low incidence of erosions, a combination of both produces extensive mucosal damage.

6 The (15S)-methyl analogue of prostaglandin $E_2$ reduced erosion formation induced by indomethacin and acid-taurocholate administration.

7 It is suggested that this protective action of the prostaglandin analogue may be linked to changes in gastric mucosal permeability and in mucosal blood flow.

## Introduction

The presence of endogenous bile in the gastric lumen has been implicated in the pathogenesis of the acute gastric lesions which follow administration of non-steroid anti-inflammatory agents (Abtahi & Djahanguiri, 1975). Furthermore, the administration of bile acids or salts into the gastric lumen greatly increases the formation of gastric mucosal erosions following intragastric or systemic administration of aspirin-like drugs in rats (Semple & Russell, 1975; Whittle, 1975). In the present study, changes in gastric mucosal blood flow and in the resistance of the mucosa to acid back-diffusion have been investigated as possible mechanisms underlying such acute erosion formation in the rat. In addition, since prostaglandin $E_2$ methyl analogues can prevent this mucosal damage (Carmichael, Nelson, Russell, Chandra, Lyon & Cochran, 1976; Whittle, 1976a), the effects of the prostaglandin analogue on these parameters have also been studied.

A preliminary account of this work has been presented to the British Pharmacological Society (Whittle, 1976b).

## Methods

### *Determination of acid back-diffusion*

The gastric lumen of the rat (anaesthetized with urethane) was perfused (0.1–0.2 ml/min) with acid-saline using the techniques described in detail by Main & Whittle (1973a). Acid back-diffusion across the mucosa was determined by titration of the gastric perfusate and the perfusing fluid with an autoburette (Radiometer, Copenhagen). The decrease in the titratable acid concentration following perfusion was expressed as acid-loss, µEq/minute.

### *Measurement of potential difference*

Potential difference across the mucosa was determined with balanced calomel electrodes (Beckman-R11C,

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Ltd.) connected by polyethylene catheters (Miniven, Portex Ltd.) filled with saturated potassium chloride solution, and agar (4% w/v) to the outflow cannula from the gastric lumen and to either the cannula in a femoral vein, or to a subcutaneous saline-filled bleb. Potential difference was continuously recorded with a millivoltmeter (Comark Electronics Ltd.) and displayed on a chart-recorder (Devices; MX2).

### *Gastric mucosal blood flow*

Changes in mucosal blood flow were determined by the [$^{14}$C]-aniline clearance technique (Main & Whittle, 1973a) and expressed as percentage of the values obtained during the control periods.

### *Assessment of gastric mucosal erosions*

The perfused stomachs were removed at the end of the experimental period, opened along the lesser curvature and pinned flat on a cork board. Erosions, which formed in the glandular mucosa, were counted and each one given a severity rating on a 1–3 scale as previously described (Main & Whittle, 1975a). The total, divided by 10, was the 'erosion index' for that stomach.

### *Drugs*

Indomethacin (Merck, Sharp & Dohme, Ltd.) was dissolved in 5% w/v sodium bicarbonate solution (pH 8) to give a final concentration of 10–20 mg/ml, and immediately injected subcutaneously. For intravenous injection, this indomethacin solution was diluted (1 volume in 4 volumes) with distilled water (an isotonic solution of sodium bicarbonate being 1.26% w/v).

Crude sodium taurocholate (Sigma Chemical Co.) dissolved in distilled water, was added to acid saline, filtered (Whatman No. 1 paper), centrifuged (1000 *g* for 5 min) and the supernatant diluted to give the desired acid and bile-salt concentration. In experiments with acid and acid-taurocholate perfusion, the acid-taurocholate solution was adjusted to give the same titre as the control acid solution. The final bile-salt concentration was expressed in terms of the sodium taurocholate content of the crude material. Prostaglandins, stored in methanol (−5°C) were made up freshly in 0.9% w/v NaCl solution (saline) following evaporation of the methanol when required and injected subcutaneously or by intravenous infusion.

### *Statistical analysis*

Results are expressed as the mean ± s.e. mean where (*n*) is the number of values in the group. The significance of the data was evaluated using Student's *t* test for paired and non-paired data and also the non-parametric Mann-Whitney U-test, where appropriate. $P<0.05$ was taken as significant.

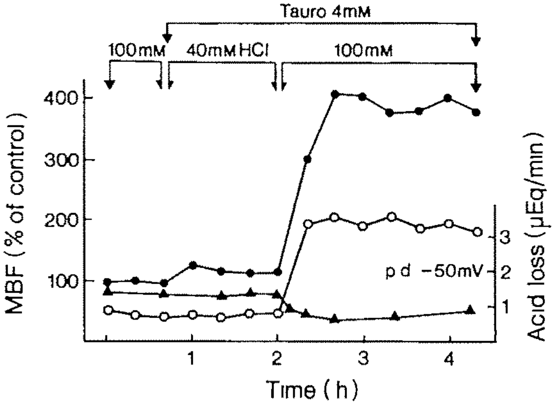

**Figure 1** Effects of acid saline (HCl, 40–100 mM) and taurocholate (Tauro, 4 mM) perfusion on gastric mucosal blood flow (MBF, ●, left ordinate scale), acid-loss (○, right ordinate scale) and potential difference (p.d., ▲, right ordinate scale).

## *Results*

### *Formation of gastric mucosal erosions*

Perfusion of the gastric lumen for 3 h with acid-saline (100 mM HCl, pH 1) did not result in damage to the mucosa. Indomethacin (20 mg/kg, s.c.), administered at the start of the acid perfusion led to a small, variable incidence of erosions after 3 h, (the erosion index was 1.1 ± 0.8, $n=3$). Gastric perfusion of the bile salt, sodium taurocholate (2 mM) in acid solution (100 mM HCl) also gave a low 3 h-erosion index (0.5 ± 0.1, $n=3$). However, as shown previously (Whittle, 1976a), administration of indomethacin at the start of the acid-taurocholate perfusion led to a significant potentiation of erosion formation (the erosion index was 6.5 ± 0.4, $n=5$; $P<0.001$).

### *Effect of bile salt on acid back-diffusion*

During gastric perfusion (0.1–0.2 ml/min) with acid saline during control periods, there was always a detectable and consistent acid back-diffusion across the mucosa (Figure 1). This resting acid back-diffusion was dependent on the acid concentration (10–100 mM HCl; 8 observations) of the perfusing fluid. The overall resting acid loss during 3 h perfusion with acid saline (100 mM HCl) was 152 ± 29 μEq/min ($n=3$).

The resting back-diffusion of acid was significantly ($P<0.001$) increased by perfusion with taurocholate

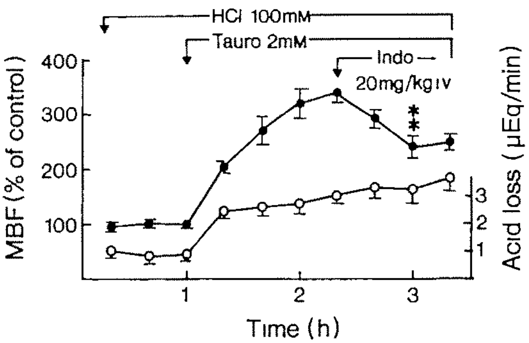

**Figure 2** Effects of indomethacin (Indo) administration (20 mg/kg, i.v.) on mucosal blood flow (MBF, ●, left ordinate scale) and acid-loss (○, right ordinate scale) during taurocholate (Tauro, 2 mM) and acid (HCl, 100 mM) perfusion. Results are mean of four experiments. Vertical lines show s.e. mean. ** $P<0.01$.

(2 mM) in acid solution (100 mM HCl), and was independent of the rate of perfusion; an increase in acid loss from 0.9 ± 0.4 to 2.5 ± 0.2 µEq/min after 1 h ($n=3$) was observed when the perfusion rate was 0.1 ml/min, and an increase from 0.9 ± 0.2 to 2.6 ± 0.3 µEq/min ($n=5$) was seen at a perfusion rate of 0.2 ml/minute. For subsequent studies, a perfusion rate of 0.2 ml/min was employed.

In a further three experiments, the acid loss appeared to be dependent both on the acid concentration (60–120 mM HCl) and the taurocholate concentration (1–10 mM). In the experiment illustrated in Figure 1, perfusion of taurocholate (4 mM) with a low acid concentration (40 mM HCl) had little effect on acid loss, whereas a marked increase was seen when the acid concentration was raised to 100 mM HCl.

### *Effect of indomethacin on acid back-diffusion*

During perfusion of acid saline (100 mM HCl), intravenous administration of indomethacin (20 mg/kg over 10 min) had no consistent effect on acid back-diffusion across the mucosa; in 4 experiments the resting acid-loss (0.93 ± 0.12 µEq/min) was similar to that 1 h after indomethacin (0.98 ± 0.13 µEq/min, $P<0.05$).

When indomethacin (20 mg/kg, s.c.) was administered at the start of gastric perfusion of taurocholate (2 mM) in acid solution, the overall loss of acid across the mucosa was 458 ± 39 µEq/3 h ($n=5$) compared with 364 ± 55 µEq/3 h ($n=5$; $P<0.05$) for taurocholate perfusion alone. In 4 other experiments where indomethacin* was injected intravenously during acid-taurocholate perfusion, there was no significant increase in the acid-loss determined at 20 min intervals for 1 h (Figure 2).

### *Changes in potential difference (p.d.)*

In eight experiments during acid-saline perfusion (pH 1), the resting p.d. (−42.3 ± 1.2 mV) rapidly fell, following perfusion of taurocholate (2 mM at pH 1) to −32.6 ± 1.5 mV after 10 min ($P<0.001$). As shown in Figure 1, the p.d. tended to return towards resting values during the continued perfusion of taurocholate.

During acid-saline perfusion, indomethacin (20 mg/kg, i.v.) had no consistent effect on p.d. (3 observations).

### *Effects of indomethacin on mucosal blood flow*

As previously observed (Main & Whittle, 1975a), indomethacin decreased resting gastric mucosal blood flow; in the present experiments during acid-saline perfusion (100 mM HCl), intravenous injection of indomethacin (20 mg/kg over 10 min) caused a significant ($P<0.01$) fall in mucosal blood flow by 20 ± 5% of the control value, after 1 h ($n=5$).

### *Effects of taurocholate on mucosal blood flow*

Perfusion of taurocholate (1–10 mM) in acid saline (60–120 mM HCl) caused a marked increase in mucosal blood flow (Figure 1 and 2) which reached steady levels within 60–80 minutes. This increase in mucosal blood flow was only seen at high acid concentrations, when acid back-diffusion occurred (Figure 1), and was therefore not due to a direct action of taurocholate on the mucosa.

In a further three experiments where either the concentration of acid (60–120 mM HCl) or taurocholate (2–10 mM) was varied, the changes in acid back-diffusion were accompanied by corresponding changes in mucosal blood flow and there appeared to be a direct relationship between these parameters.

### *Effects of taurocholate and indomethacin on mucosal blood flow*

In four experiments, when steady rates of mucosal blood flow and acid-loss were obtained during perfusion of taurocholate (2 mM) and acid (100 mM HCl), indomethacin (20 mg/kg, i.v.) was administered (Figure 2). Although there was no marked change in acid-loss, a significant decrease in the mucosal blood flow was observed ($P<0.01$ after 40 minutes). The effect of indomethacin and taurocholate on mucosal blood flow and acid-loss are shown in Figure 3.

### *Effects of a prostaglandin methyl analogue*

Subcutaneous injections of (15S)-15 methyl prostaglandin $E_2$ methyl ester (5 µg/kg every hour) from the start of taurocholate (2 mM) and indomethacin administration (20 mg/kg, s.c.) during acid perfusion (100 mM HCl) caused a significant ($P<0.02$)

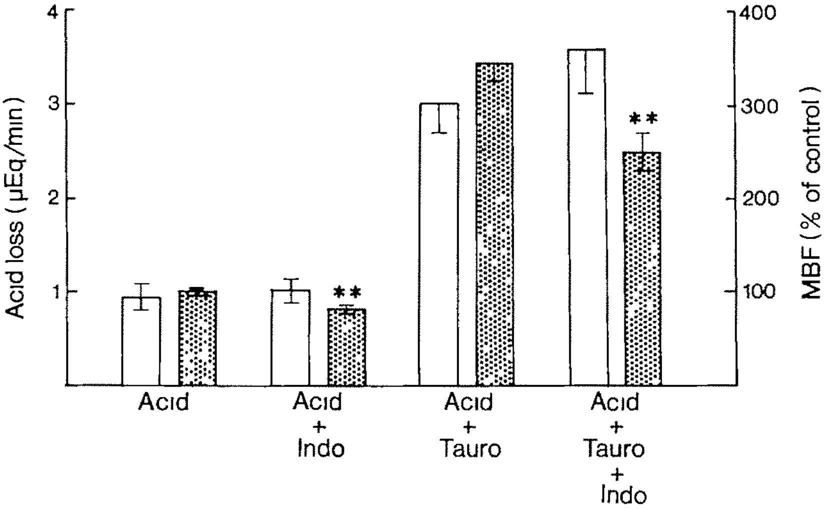

**Figure 3** Summary of the changes in acid back-diffusion (open columns) and mucosal blood flow (MBF, stippled columns) during gastric perfusion of acid saline (HCl, 100 mM). Results, expressed as the mean are the values 1 h after administration of either indomethacin (Indo, 20 mg/kg i v ) in four experiments, or successive treatments with taurocholate (Tauro, 2 mM) and indomethacin (20 mg/kg, i v.) in a further four experiments. Vertical lines show s.e. mean.

reduction in the erosion index observed after 3 h (from 6.5 ± 0.4 to 2.9 ± 0.5). The overall acid-loss during this 3 h perfusion period was also significantly reduced from 458 ± 39 µEq/3 h ($n=5$) to 345 ± 25 µEq/3 h ($n=4$; $P<0.02$).

In eight other experiments with indomethacin (20 mg/kg, s.c.) and taurochlolate (2 mM)-administration during 60 mM HCl perfusion, the 3 h-erosion index was reduced from 3.9 ± 0.3 to 1.0 ± 0.4, and the acid loss from 262 ± 9 to 206 ± 16 µEq/3 h ($n=4$ for each group; $P<0.01$) by concurrent administration of the (15S)-15 methyl prostaglandin $E_2$ analogue (5 µg $kg^{-1}$ $h^{-1}$, s.c.).

In further experiments, the effects of intravenous infusion of (15S)-15 methyl prostaglandin $E_2$ on mucosal blood flow and p.d. were determined during acid (100 mM HCl) perfusion. When steady rates of mucosal blood flow were obtained, 1.5 h following indomethacin administration, infusion of (15S)-15 methyl $E_2$ (5 µg $kg^{-1}$ $h^{-1}$) caused a gradual increase in p.d. (by 7.1 ± 1.5 mV after 1 h, $n=5$), and an increase in mucosal blood flow (by 38.3 ± 7.3% of control after 1 h, $n=5$). In three other experiments during concurrent acid-taurocholate perfusion, this dose of the prostaglandin analogue likewise increased mucosal blood flow (by 40 ± 8% of control) and p.d. (by 5.2 ± 0.6 mV).

During acid perfusion alone, an increase in p.d. (8 mV, 1 experiment) was observed following the prostaglandin infusion; in three experiments, initial single intravenous injections of (15S)-15 methyl $E_2$ (2.5 µg/kg) also increased the resting p.d. (by 8.7 ± 0.9 mV after 30 min, $n=3$) although subsequent repeated injections gave variable results (a fall in p.d. was often observed).

## Discussion

The reflux of bile into the gastric lumen has been considered as a possible factor in the aetiology of peptic ulceration (Du Plessis, 1965) and in the pathogenesis of the gastric erosions induced by non-steroid anti-inflammatory agents (Djahanguiri, Abtahi & Hemmati, 1973; Semple & Russell, 1975). Bile, by virtue of its surface-active properties, is thought to damage the gastric mucosa by promoting the back-diffusion of hydrogen-ions from the lumen across the mucosa (Davenport, 1968). However, in the present study the increase in acid back-diffusion with the bile salt, taurocholate, in acid solution did not lead to marked mucosal damage. It is therefore possible that the accompanying rise in gastric mucosal blood flow is a protective mechanism preventing the excessive accumulation of acid within the mucosa under such pathological conditions. This is supported by the finding that indomethacin, in doses which reduced this hyperaemic response to taurocholate perfusion, caused a marked potentiation of erosion formation.

An increase in mucosal blood flow, measured by aminopyrine clearance, has also been observed in the exteriorized, chambered preparation of canine fundic segments, using concentrations of taurocholate (5 mM) in acid solution in the same range as those in the present study (Ritchie, 1975). In a similar preparation, a high concentration of taurocholate (40 mM), which induced a ten-fold increase in acid back-diffusion with gross mucosal damage, also increased mucosal blood flow as measured by microspheres (Cheung, Moody & Reese, 1975). However, in that study aminopyrine clearance failed to show any marked change, and the authors suggested that this latter technique had limitations under such conditions of extensive mucosal damage. Although we have used a similar clearance technique to estimate changes in rat mucosal blood flow, the results were obtained at the early stages of erosion formation or with only slight mucosal damage. It is not known whether such mucosal damage could explain the divergent results of O'Brien & Silen (1973), who found that high concentrations of taurocholate (10–40 mM) caused a decrease in aminopyrine clearance. This action, observed even in the absence of acid back-diffusion, suggests some direct effect of the bile-salt preparation used in their study and contrasts with the present study where taurocholate had no direct effect on mucosal blood flow independent of acid back-diffusion. The bulk of the evidence in the dog, therefore, agrees with the present findings in the rat that acid back-diffusion can lead to an increase in mucosal blood flow. The mechanism by which this mucosal hyperaemia occurs is not known, but it may be a direct response of the mucosal microvasculature to the increased acid back-diffusion, or to the local release of mediators, possibly a prostaglandin.

The fall in gastric mucosal blood flow induced by indomethacin, in doses sufficient to inhibit postaglandin formation (Vane, 1971) in the mucosa (Main & Whittle, 1975a) could suggest a role for endogenous prostaglandins (or some other product of the prostaglandin cyclo-oxygenase system) in the local regulation of the gastric microcirculation; it has been found that primary prostaglandins of the E and A series for example, can increase resting mucosal blood flow (Main & Whittle, 1973b). Whether the reduced local blood flow with indomethacin is solely the consequence of a reduction in a prostaglandin-mediated vasodilator tone is not known. It is possible that cyclo-oxygenase inhibition by indomethacin leads to the production of vasoconstrictor metabolites from prostaglandin precursors, or that indomethacin has inherent vasoconstrictor activity. The greater activity of indomethacin on taurocholate-elevated mucosal blood flow could also suggest that microcirculatory regulation by the prostaglandin-system is of more importance under patho-physiological conditions.

Although these studies can give an indication of the gross changes in the local microcirculation, the measurement of mucosal blood flow by the techniques currently available can give only limited information about microvascular events at the localized sites of erosion formation. Small overall changes could therefore reflect intense focal ischaemia and such areas would be likely to be the sites of subsequent erosions, especially in the presence of other noxious stimuli, such as acid.

The failure of indomethacin, administered systemically, to alter either p.d., which can give an indication of mucosal hydrogen- or sodium-ion flux, or the acid back-diffusion, may argue against a local role for endogenous prostaglandins in the maintenance of the rat mucosal 'barrier'. However, intragastric administration of several non-steroid, anti-inflammatory agents including indomethacin, has previously been demonstrated to promote acid-loss and changes in p.d. in the dog (Chvasta & Cooke, 1972). Although this could reflect a species difference, a more likely explanation is that such drugs have a topical action, perhaps of a physico-chemical nature, unrelated to inhibition of local prostaglandin synthesis. The possible effects of these aspirin-like drugs on gastric mucosal blood flow are therefore complex with both direct effects and those as a consequence of acid back-diffusion, and the observed actions could well depend on the dose and route of administration. Furthermore, any hyperaemic response to acid back-diffusion caused by these aspirin-like drugs may be attenuated by their concurrent direct effects on the microvasculature. Thus, the potency of non-steroid, anti-inflammatory agents in causing either or both actions may give a good indication of the ability of such drugs to cause gastric erosions.

These results in the rat suggest that, whereas a reduction in mucosal blood flow, as seen with indomethacin, or an increase in acid back-diffusion, as observed with taurocholate, can lead to a low incidence of erosions, a combination of both agents and both actions produces extensive mucosal damage. A similar conclusion regarding the effects of mucosal ischaemia and acid back-diffusion was reached from studies on the dog mucosa (Ritchie, 1975), which showed that, whereas the reduction in mucosal blood flow with vasopressin alone did not produce erosions, the mucosal damage with taurocholate in acid solution was potentiated. It is also of interest that in an early study on the dog, haemorrhagic shock (which could reduce gastric blood flow) produced mucosal lesions only in the presence of both acid test-solution and bile-salts (Safaie-Shirazi, Denbesten & Hamza, 1972).

A 15-methyl analogue of prostaglandin $E_2$ was previously found to inhibit the gastric erosions induced by taurocholate and a non-steroid anti-inflammatory agent in the rat (Carmichael *et al.*, 1976). However, in that study the prevention of mucosal damage by intragastric administration of the (15R)-epimer, which could be converted to the potent gastric antisecretory (15S)-epimer in the acid environment (see Main & Whittle, 1975b), is likely to be the consequence of inhibition of the gastric acid secretion (Whittle, 1976a). The ability of the (15S)-methyl prostaglandin $E_2$ analogue to inhibit erosion formation in the presence of exogenous perfused acid (Whittle, 1976a) suggests further protective mechanisms of the prostaglandins on the mucosa.

The observation that the prostaglandin methyl analogue reduced the acid loss provoked by taurocholate supports the findings in the dog that the analogue can reduce the acid back-diffusion following intragastric administration of aspirin and indomethacin (Cohen, 1976). Furthermore, this prostaglandin analogue also increased rat mucosal blood flow under conditions of taurocholate and indomethacin administration, as shown previously under resting conditions (Main & Whittle, 1975b). It is likely that such actions on the mucosal permeability to acid (or other ions), coupled with the observed changes in mucosal blood flow are important factors in the process by which prostaglandins prevent gastric mucosal damage.

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# STUDIES ON THE CARDIOVASCULAR EFFECTS OF PINDOLOL IN DOCA/SALINE HYPERTENSIVE RATS

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1 A hypotensive response to orally administered pindolol in conscious normotensive and deoxycorticosterone acetate (DOCA)/saline hypertensive rats (DS-rats) is described. In DS-rats, pindolol (10–50 μg/kg) produced a dose-dependent fall in blood pressure and elevation of resting heart rate.

2 The hypotensive response and tachycardia produced by oral pindolol (50 μg/kg) in DS-rats were prevented by propranolol (5 mg/kg), suggesting that pindolol's effects are mediated by $\beta$-adrenoceptor stimulation.

3 After mecamylamine (10 mg/kg), oral pindolol (50 μg/kg) produced a further fall in blood pressure in DS-rats, suggesting that its hypotensive effects are probably mediated in the peripheral vasculature.

4 Pretreatment with oral pindolol (10 or 50 μg/kg) resulted in a reduction of neuronally-induced tachycardia in pithed DS-rats; neuronally-evoked pressor effects were also antagonized by pindolol (50 μg/kg, orally).

5 Whereas pindolol, 50 μg/kg orally or intraperitoneally, produced a marked and progressive hypotensive response of rapid onset (20 min) in DS-rats the same dose intravenously produced a smaller response of delayed onset (80 minutes).

6 In anaesthetized DS-rats, an equivalent degree of cardiac $\beta$-adrenoceptor blockade was produced by pretreatment with pindolol, 50 μg/kg orally (2 h previously) or intravenously (1 h previously).

7 After administration of pindolol, 2 mg/kg intravenously, to conscious DS-rats, the tachycardia produced by intravenous isoprenaline, 3 μg/kg, was almost abolished for the first 60 min of the study, whereas a hypotensive response to pindolol was delayed in onset (100 minutes).

8 The hypotensive response and tachycardia produced by oral pindolol, 50 μg/kg, in DS-rats were prevented by inhibition of metabolic enzyme activity by pretreatment with Proadifen (SKF 525-A), 80 mg/kg.

9 The results suggest that pindolol's effects on blood pressure and heart rate in the conscious DS-rat are mediated by a metabolite(s) acting by stimulation of peripheral $\beta$-adrenoceptors.

## Introduction

Pindolol is a potent, non-selective $\beta$-adrenoceptor blocking drug (Giudicelli, Schmitt & Boissier, 1969). possessing intrinsic sympathomimetic activity (Barrett & Carter, 1970). Like many other $\beta$-adrenoceptor blocking drugs (see review by Simpson, 1974), pindolol is an effective anti-hypertensive drug in man (e.g. Feltham, Watson, Peel, Dunlop & Turner, 1972; Thorpe, 1972; Van Coller, 1973; Morgan, Sabto, Anavekar, Louis & Doyle, 1974; Stokes, Weber & Thornell, 1974) although a pressor response has been reported in some patients (Collins & King, 1972; Morgan, Louis, Dawborn & Doyle, 1972; Waal-Manning & Simpson, 1975).

In laboratory studies prolonged oral or subcutaneous administration of pindolol has been shown to reduce the blood pressure of spontaneously hypertensive and normotensive rats (Garvey & Ram, 1975), although the course of development of renal or deoxycorticosterone acetate (DOCA)/saline (DS) hypertension is apparently not influenced by prolonged oral dosage (Takeda, Sakurai & Imai, 1975). In the rabbit, a small acute reduction in blood pressure has been reported following the intravenous administration of pindolol (Weber, Thornell & Stokes, 1974).

In the experiments described here we have studied the cardiovascular effects of pindolol in normotensive and DS-rats using single oral doses that are appreciably lower than those hitherto used in rats (Garvey & Ram, 1975; Takeda *et al.*, 1975). Furthermore, in the knowledge that pindolol is extensively metabolized in rats (Kiechel, Niklaus, Schreier & Wagner, 1975), we have investigated the possibility that the acute cardiovascular responses observed following pindolol administration are dependent upon metabolic activation of the parent drug.

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## Methods

Male Sprague-Dawley rats were used for these studies.

### *Induction of experimental hypertension*

Rats, 120–150 g, were made hypertensive by unilateral nephrectomy, subcutaneous implantation of 2 compressed tablets each containing 25 mg deoxycorticosterone acetate (DOCA) and replacement of drinking water by 0.9% w/v NaCl solution (saline) until experimental use 4–6 weeks later.

### *Implantation of catheters for the direct measurement of arterial blood pressure and the intravenous administration of drugs*

Polythene catheters were implanted, under ether anaesthesia, in the abdominal aorta for direct blood pressure measurement, and also in the abdominal vena cava of some animals to facilitate intravenous drug administration. The operative procedure has been previously described (Buckingham, 1976). The rats were allowed 1 to 2 days to recover before use. Normotensive rats used for these experiments weighed 290–360 grams.

### *Direct measurement of arterial blood pressure and heart rate in conscious rats*

Groups of 7–13 rats were used for each treatment. Blood pressure was recorded in conscious unrestrained animals with a Statham P23 Db pressure transducer (1 mmHG≈133 Pa) connected to a Grass (Model 7) Polygraph. Heart rate was recorded either from a Grass tachograph, triggered by the arterial pulse pressure wave, or was counted from the record after increasing the chart speed.

### *Drug administrations in conscious rats*

(a) The effects of single doses of pindolol, 10, 25 or 50 μg/kg orally were studied in normotensive and DS-rats. Mean arterial blood pressure and heart rate were recorded immediately before dosing and then at hourly intervals for 4 hours.

(b) The effects of pindolol, 50 μg/kg orally, and propranolol, 5 mg/kg intraperitoneally both alone and in combination were studied in DS-rats. Blood pressure and heart rate were monitored as in (a).

(c) The effects of pindolol, 50 μ/kg orally, and mecamylamine, 10 mg/kg intraperitoneally, both alone and in combination were studied in DS-rats. Blood pressure and heart rate were monitored as in (a).

(d) The effects of pindolol, 50 μg/kg orally, intraperitoneally or intravenously, were studied in DS-rats. Blood pressure and heart rate were recorded immediately before dosing and then at 20 min intervals for 2 hours.

(e) The effect of pindolol, 2 mg/kg intravenously was studied in DS-rats. Blood pressure was monitored as in (d). In a separate experiment the change in heart rate produced by isoprenaline, 3 μg/kg intravenously was determined immediately before dosing with pindolol, 2 mg/kg intravenously, and then at 20 min intervals for 2 hours.

(f) The effect of pindolol, 50 μg/kg orally, was studied in DS-rats following treatment with Proadifen (SKF 525-A), 80 mg/kg intraperitoneally, 4 h previously. Blood pressure and heart rate were recorded immediately before dosing with pindolol and then at 20 min intervals for 2 hours.

In all experiments the changes in cardiovascular parameters were related to the values obtained immediately before dosing (zero time).

### *Stimulation of sympathetic outflow in pithed rats*

DS-rats received pindolol, 10 or 50 μg/kg orally, or vehicle 2 h before the start of frequency-response determinations. Animals were prepared in pairs for stimulation of the entire sympathetic outflow by the method of Gillespie & Muir (1967). The 3 treatments were randomized throughout the group of 30 rats. Each animal received intravenous atropine, 1 mg/kg, tubocurarine, 2 mg/kg, and heparin, 100 units/kg, after pithing, Preparations were electrically stimulated for periods of 20 s every 8 min by monophasic, square-wave pulses of 0.5 ms duration at supramaximal voltage over the frequency range 0.25–8 hertz. Changes in diastolic blood pressure were recorded from the left carotid artery with a Statham P23 Db pressure transducer connected to a Grass Polygraph; heart rate was recorded with a Grass tachograph.

### *Assessment of β-adrenoceptor blocking activity in anaesthetized rats*

DS-rats received pindolol, 50 μg/kg orally or intravenously, or appropriate vehicle treatment. At 45 min after intravenous treatment or 105 min after oral dosing, the animals were anaesthetized with pentobarbitone sodium, 75 mg/kg intraperitoneally and prepared for recording blood pressure and heart rate. Fifteen minutes later changes in heart rate were recorded in response to isoprenaline, 0.3, 1, 3 and 10 μg/kg intravenously. Dose-response curves to isoprenaline were completed within 30 min in each animal.

### *Drugs*

The following drugs were used: isoprenaline sulphate (Burroughs Wellcome); pindolol (Sandoz); Proadifen ($\beta$-diethyl-aminoethyl-diphenylpropylacetate hydrochloride) (Smith, Kline and French); atropine sulphate (BDH); heparin (Roche); mecamylamine hydrochloride (Merck, Sharp and Dohme); propranolol hydrochloride (ICI) and (+)-tubocurarine chloride BP (BDH).

Doses of atropine sulphate, pindolol and isoprenaline sulphate are expressed as base equivalents. Doses of propranolol hydrochloride, mecamylamine hydrochloride and (+)-tubocurarine chloride are expressed as the salts.

Pindolol was suspended in water for oral administration, and dissolved in acidified saline (pH 5) for intravenous injection. Other drugs were dissolved in saline.

### *Statistical analysis*

Data were analysed by Student's *t* test; *P* values $<0.05$ were considered significant.

## Results

### *Effect of oral pindolol on the blood pressure and heart rate of conscious normotensive rats*

Pindolol, 10 or 25 µg/kg did not significantly alter blood pressure, whilst 50 µg/kg produced a significant hypotension (11–16 mmHg) for 4 hours. Pindolol, 10 µg/kg, did not alter heart rate in normotensive rats, but 25 µg/kg produced a significant tachycardia (29–33 beats/min) at 1, 2 and 4 h after dosing, and 50 µg/kg evoked a more marked response (47–54 beats/min) for 4 hours.

### *Effect of oral pindolol on the blood pressure and heart rate of conscious DS-rats*

In DS-rats pindolol, 0.5 or 2.5 µg/kg, did not significantly affect blood pressure or heart rate. A significant and dose-dependent hypotension was produced by pindolol at doses of 10 µg/kg (1–3 h), 25 µg/kg (1–4 h), and 50 µg/kg (1–4 h) (Figure 1a). In the same studies pindolol, 10 µg/kg, did not significantly alter heart rate whilst 25 or 50 µg/kg significantly increased heart rate for 3 h after dosing (Figure 1b).

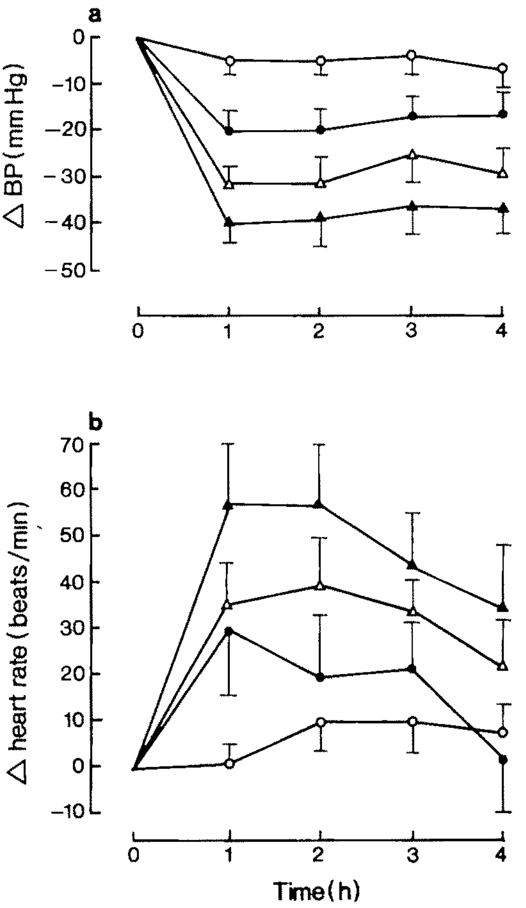

**Figure 1** Time course (h) of change in (a) mean arterial blood pressure (ΔBP mmHg) and (b) heart rate (beats/min) of conscious DS-rats, after pindolol, 10 µg/kg (●, $n=12$), 25 µg/kg (Δ, $n=13$), 50 µg/kg (▲, $n=13$) orally or control vehicle (O, $n=13$). Vertical bars show s.e. mean; *n* is the number of animals per group The resting mean blood pressure and heart rate of each group were, respectively, for pindolol 10 µg/kg 160 ± 4 mmHg and 375 ± 10 beats/min; for 25 µg/kg 175 ± 6 mmHg and 372 ± 7 beats/min; for 50 µg/kg 173 ± 4 mmHg and 379 ± 6 beats/min; and for the control group, 166 ± 5 mmHg and 368 ± 6 beats/minute.

### *Effect of propranolol on the blood pressure and heart rate response evoked by oral pindolol in conscious DS-rats*

Simultaneously administered propranolol, 5 mg/kg intraperitoneally, abolished the fall in blood pressure produced by pindolol, 50 µg/kg, between 1 and 4 h (Figure 2a) and also prevented the significant tachycardia produced by pindolol 1 h after administration (Figure 2b). Compared with control DS-rats, propranolol significantly reduced heart rate between 1 and 4 h and significantly raised blood pressure 1 h after dosing (Figure 2).

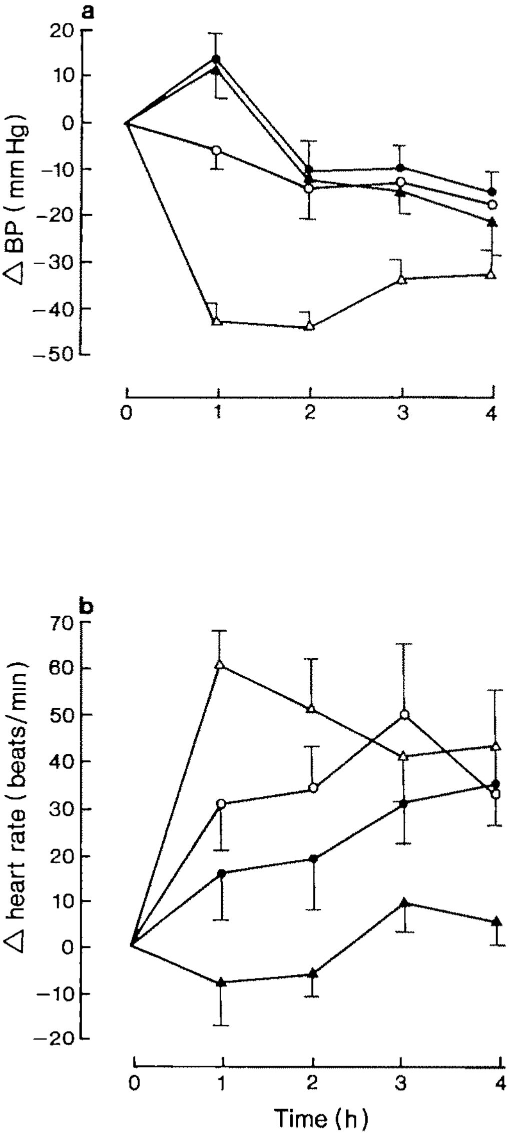

**Figure 2** Time course (h) of change in (a) mean arterial blood pressure (ΔBP mmHg) and (b) heart rate (beats/min) of conscious DS-rats after pindolol, 50 μg/kg orally (△, $n=9$), propranolol, 5 mg/kg intraperitoneally (▲, $n=8$), propranolol, 5 mg/kg intraperitoneally + pindolol, 50 μg/kg orally (●, $n=9$) or control vehicle (○, $n=7$). Vertical bars show s.e. mean; $n$ is the number of animals per group. The resting mean blood pressure and heart rate of each group were respectively, for 50 μg/kg pindolol, 162 ± 1 mmHg and 354 ± 9 beats/min; for 5 mg/kg propranolol, 160 ± 5 mmHg and 355 ± 4 beats/min; for the drug combination group, 160 ± 3 mmHg and 342 ± 8 beats/min: and for the control group, 165 ± 3 mmHg and 347 ± 9 beats/minute.

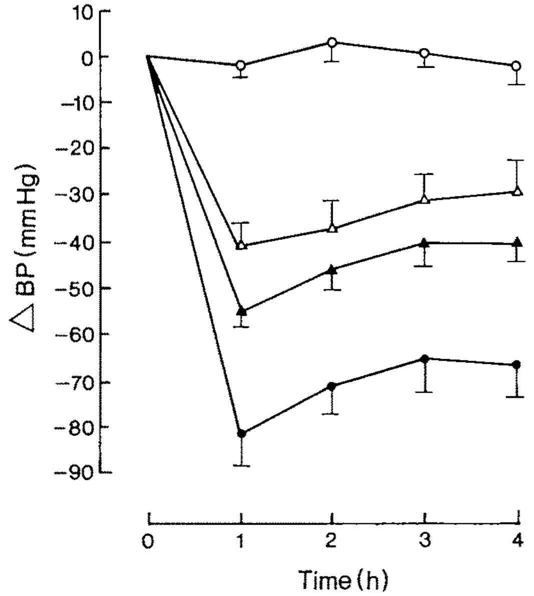

**Figure 3** Time course (h) of change in mean arterial blood pressure (ΔBP mmHg) of conscious DS-rats after pindolol, 50 μg/kg orally (△, $n=10$), mecamylamine, 10 mg/kg intraperitoneally (▲, $n=10$), mecamylamine, 10 mg/kg intraperitoneally + pindolol, 50 μg/kg orally (●, $n=9$) or control vehicle (○, $n=9$). Vertical bars show s.e. mean; $n$ is the number of animals per group. The resting mean blood pressure of each group were respectively, for 50 μg/kg pindolol, 145 ± 9 mmHg; for 10 mg/kg mecamylamine, 139 ± 7 mmHg for the drug combination group, 141 ± 9 mmHg; and for the control group, 140 ± 8 mmHg

### *Effect of mecamylamine on the blood pressure response evoked by oral pindolol in conscious DS-rats*

The hypotensive response which followed simultaneous administration of mecamylamine, 10 mg/kg intraperitoneally, and pindolol, 50 μg/kg, was significantly greater than that produced by either drug alone over the entire 4 h time course (Figure 3).

### *Effect of oral pindolol pretreatment on neuronally-evoked tachycardia and pressor responses in pithed DS-rats*

*Heart rate responses.* Treatment with pindolol, 10 or 50 μg/kg 2 h previously, significantly reduced the frequency-dependent tachycardia evoked by stimulation of the sympathetic outflow from the spinal cord of pithed DS-rats (Figure 4a).

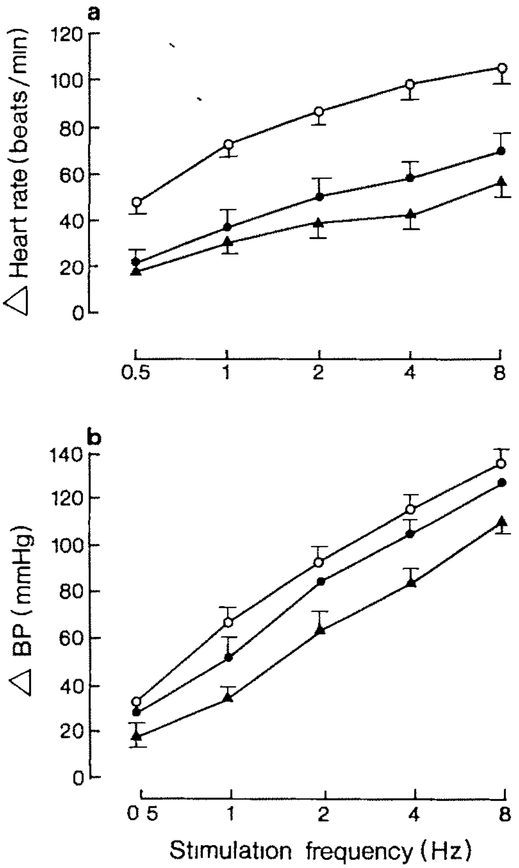

**Figure 4** Relationship between frequency of stimulation (Hz) of the spinal cord of pithed DS-rats and (a) increase in heart rate (beats/min) and (b) increase in diastolic blood pressure (ΔBP mmHg) in animals treated 2 h previously with oral pindolol, 10 µg/kg (●), 50 µg/kg (▲), or control vehicle (○). Vertical bars show s.e. mean; 10 animals were used for each group.

**Table 1** Effects of oral pindolol treatment (2 h previously) on the basal heart rate and diastolic blood pressure of pithed DS-rats

| *Pretreatment* | *Basal heart rate (beats/min)* | *Basal diastolic blood pressure (mmHg)* |
|---|---|---|
| Control ($n=10$) | 341 ± 7 | 43 ± 3 |
| Pindolol 10 µg/kg ($n=10$) | 383 ± 14 | 41 ± 5 |
| 50 µg/kg ($n=10$) | 390 ± 9 | 32 ± 2 |

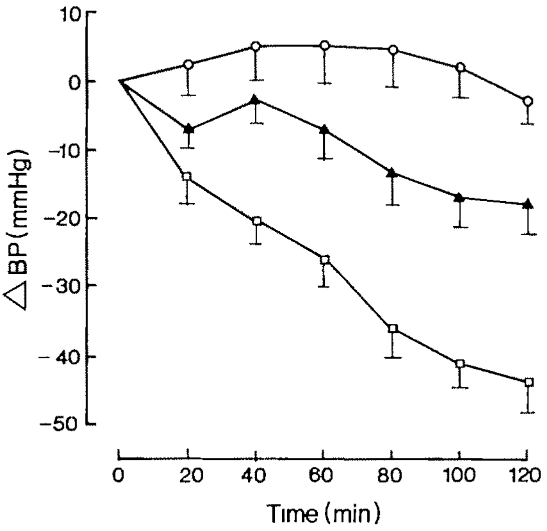

**Figure 5** Time course (min) of change in mean arterial blood pressure (ΔBP mmHg) of conscious DS-rats after pindolol, 50 µg/kg intravenously (▲), 50 µg/kg orally (□), or control vehicle (○). Vertical bars show s.e. mean. Groups of 9 rats were used. The resting mean arterial blood pressure and heart rate of each group were for pindolol, 50 µg/kg intravenously, 167 ± 5 mmHg and 375 ± 12 beats/min; for pindolol 50 µg/kg orally, 181 ± 5 mmHg and 379 ± 25 beats/min; and for controls, 168 ± 3 mmHg and 414 ± 15 beats/minute.

*Pressor responses.* Pretreatment with pindolol, 50 µg/kg, significantly reduced the increase in diastolic blood pressure produced by sympathetic stimulation in pithed DS-rats. Pindolol, 10 µg/kg, produced no significant effect (Figure 4b).

### *Effect of oral pindolol pretreatment on the basal heart rate and blood pressure of pithed DS-rats*

The basal heart rate of pithed DS-rats was significantly elevated and the basal diastolic blood pressure significantly reduced 2 h after treatment with pindolol, 10 or 50 µg/kg (Table 1).

### *Effect of route of administration on the cardiovascular effects of pindolol in conscious DS-rats*

Pindolol, 50 µg/kg orally, produced a significant fall in mean arterial blood pressure (16 mmHg) within 20 min of administration. The magnitude of the response increased with time, a reduction of 40–43 mmHg occurring 80–120 min after dosing (Figure 5). In contrast, pindolol, 50 µg/kg intravenously, was much less effective in lowering blood pressure (Figure 5). The onset of the hypotensive

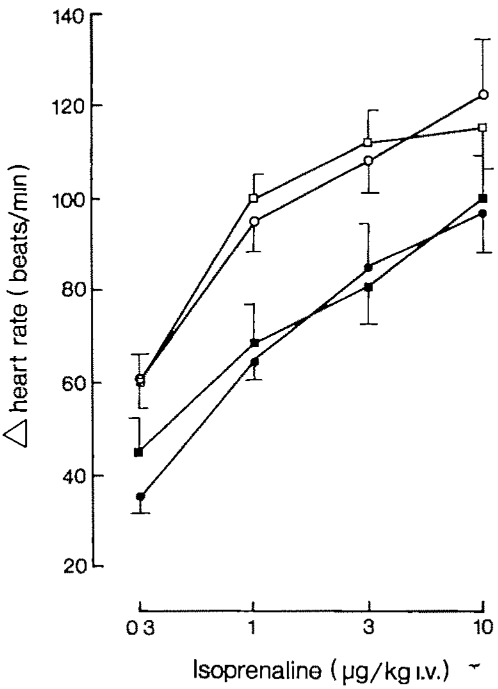

**Figure 6** Relationship between dose of isoprenaline (μg/kg i.v.) and increase in heart rate (beats/min) in anaesthetized DS-rats after intravenous control vehicle (○) or intravenous pindolol, 50 μg/kg (●); oral vehicle (□) or oral pindolol, 50 μg/kg (■). Vertical bars show s.e. mean; 7 or 8 animals per group were used for the intravenous study and 10 or 12 animals per group for the oral study.

response was considerably delayed, and a small but significant fall in blood pressure (14–19 mmHg) was observed at 80–120 minutes. The hypotensive response to pindolol, 50 μg/kg intraperitoneally, was similar in time course and magnitude to that produced by this dose orally.

Pindolol, 50 μg/kg orally, produced a significant tachycardia (67 beats/min) at 120 min, whilst 50 μg/kg intravenously significantly raised heart rate at 80 min (50 beats/min) and 100 min (67 beats/minute).

### *Degree of cardiac β-adrenoceptor blockade produced by pindolol in anaesthetized DS-rats*

The degree of cardiac β-adrenoceptor blockade, as estimated by a parallel shift in the isoprenaline (0.3–10 μg/kg intravenously) log dose-response curve, was similar following treatment with pindolol (50 μg/kg) orally (2 h previously) or intravenously (1 h previously) (Figure 6).

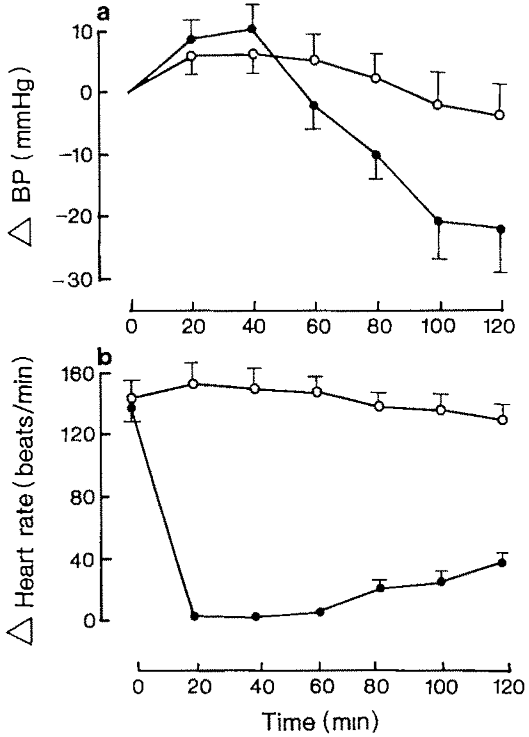

**Figure 7** (a) Time course (min) of change in mean arterial blood pressure (ΔBP mmHg) of conscious DS-rats after pindolol, 2 mg/kg intravenously (●), or control vehicle (○). Vertical bars show s.e. mean. Groups of 12 rats were used. The resting mean arterial blood pressure and heart rate of each group were, for pindolol, 2 mg/kg, 178 ± 7 mmHg and 413 ± 13 beats/min, and for controls 180 ± 5 mmHg and 413 ± 11 beats/minute. (b) Time course (min) of increase in resting heart rate produced by isoprenaline, 3 μg/kg intravenously, in controls (○, $n = 11$) and in animals treated with pindolol, 2 mg/kg intravenously (●, $n = 13$). Vertical bars show s.e. mean; $n$ is the number of animals per group.

### *Time course of changes in blood pressure, heart rate and degree of cardiac β-adrenoceptor blockade following intravenous pindolol in conscious DS-rats*

Following an initial latent period pindolol, 2 mg/kg, produced a significant fall in mean arterial blood pressure (18–19 mmHg) 100–120 min after injection (Figure 7a), without a significant change in heart rate.

In a separate experiment pindolol, 2 mg/kg, produced almost total inhibition of the tachycardia due to isoprenaline, 3 μg/kg intravenously. This antagonism of cardiac β-adrenoceptor stimulation was apparent 20 min after pindolol administration and showed only partial recovery 100 min later (Figure 7b).

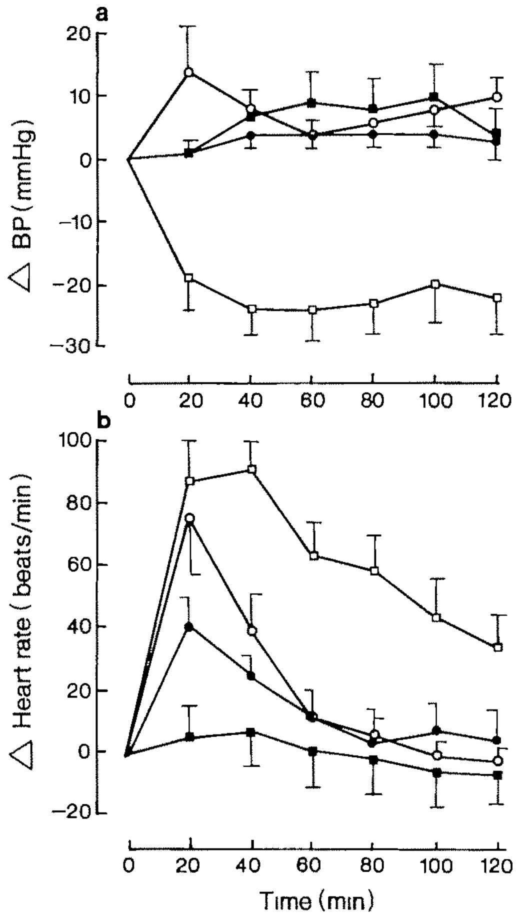

**Figure 8** Time course (min) of change in (a) mean arterial blood pressure (ΔBP mmHg) and (b) heart rate (beats/min) of conscious DS-rats. After treatment with Proadifen, 80 mg/kg intraperitoneally, 4 h previously; pindolol, 50 µg/kg orally (■), control vehicle (●). After vehicle pretreatment; pindolol 50 µg/kg orally (□), control vehicle (○). Vertical bars show s.e. mean Groups of 9 rats were used. The resting mean arterial blood pressure and heart rate of each group were as follows: after Proadifen pretreatment: pindolol, 145 ± 9 mmHg and 351 ± 10 beats/min; controls, 148 ± 10 mmHg and 347 ± 10 beats/min; after vehicle pretreatment: pindolol, 131 ± 6 mmHg and 369 ± 13 beats/min; controls, 134 ± 7 mmHg and 340 ± 6 beats/minute.

### *Effect of Proadifen (SKF 525-A) pretreatment on the cardiovascular effects of oral pindolol in conscious DS-rats*

In vehicle-pretreated DS-rats pindolol, 50 µg/kg, significantly reduced mean arterial blood pressure (28–33 mmHg) over the entire 2 h time course (Figure 8a). In addition, heart rate was significantly elevated (31–53 beats/min) in these animals between 40 min and 2 h after pindolol administration (Figure 8b). After treatment with Proadifen, 80 mg/kg intraperitoneally 4 h previously, the hypotensive response to pindolol was prevented and no tachycardia was observed. Indeed, pindolol produced a significant net reduction in heart rate (36 beats/min) 20 min after administration in Proadifen pretreated rats.

## Discussion

In acute studies pindolol (10, 25 or 50 µg/kg orally) dose-dependently reduced mean arterial blood pressure and elevated resting heart rate in conscious DS-rats. A fall in blood pressure was also observed in normotensive rats treated with pindolol, 50 µg/kg orally, suggesting that the drug's activity is hypotensive in nature and independent of any aetiological factor(s) specific to hypertensive rats. In DS-rats propranolol prevented the pindolol-induced tachycardia and hypotension, suggesting that these cardiovascular effects are mediated by $\beta$-adrenoceptor stimulation. Furthermore, in the presence of ganglion blockade produced by mecamylamine, pindolol induced a further fall in arterial blood pressure, indicating that the drug's hypotensive action is mediated post-ganglionically. These results are consistent with the hypothesis that the effects on blood pressure and heart rate produced by orally administered pindolol in DS-rats are due to partial agonist activity at vascular $\beta_2$-adrenoceptors (subserving vasodilatation) and cardiac $\beta_1$-adrenoceptors respectively. Partial agonist activity of pindolol at cardiac $\beta$-adrenoceptors has also been recognized by Barrett & Carter (1970). Results of studies in the pithed DS-rat are consistent with the observations in conscious animals. Hence a vasodilator effect probably accounts for the observed reductions in basal diastolic pressure and in neuronally-evoked pressor responses in pithed rats pretreated with pindolol, 50 µg/kg orally. Furthermore pindolol (10 or 50 µg/kg orally) increased the basal heart rate of pithed DS-rats and reduced the neuronally-evoked tachycardia, suggesting the presence of both partial agonist activity and measurable cardiac $\beta$-adrenoceptor blocking activity. However, the apparent degree of cardiac $\beta$-adrenoceptor blockade may have been exaggerated as a result of the raised resting heart rate following pindolol pretreatment.

The magnitude and time course of pindolol's hypotensive effect were dependent upon route of drug administration. When pindolol, 50 µg/kg, was adminstered orally or intraperitoneally to conscious DS-rats, a marked and progressive hypotensive response of rapid onset was produced. In contrast, the same dose given intravenously resulted in a smaller hypotensive effect with a delayed onset. This difference in the pattern of hypotensive response could not be attributed to a difference in the degree of $\beta$-adrenoceptor blockade since in anaesthetized DS-rats, pretreatment with pindolol, 50 µg/kg orally (2 h previously) or intravenously (1 h previously) resulted in a similar degree of cardiac $\beta$-adrenoceptor blockade by either route. This independence between the time-course of the hypotensive response and $\beta$-adrenoceptor blocking activity was further illustrated following intravenous injection of pindolol, 2 mg/kg, to DS-rats. Once again the hypotensive effect of pindolol was delayed in onset (100 min), whereas the heart rate response to a standard dose of isoprenaline was virtually abolished throughout the first 60 min in a parallel study. These results are consistent with the suggestion that pindolol itself is devoid of hypotensive activity in DS-rats, and that metabolic activation of the drug is required for the expression of its effects on arterial blood pressure and heart rate in this model. The observation that the hypotension and tachycardia normally associated with oral pindolol administration were prevented by pretreatment with Proadifen, an inhibitor of metabolic enzymes, adds further support to this hypothesis. The results suggest, therefore, that whilst pindolol and probably the active metabolite(s) possess $\beta$-adrenoceptor blocking properties, the hypotensive activity resides solely in the metabolite(s).

The literature contains a puzzling lack of uniformity with respect to the proposed partial agonist activity of pindolol. Whilst the drug has been shown to possess sympathomimetic activity in rabbit isolated atria (Lubawski & Wale, 1969) and in catecholamine-depleted rat hearts *in vivo* (Barrett & Carter, 1970), no such properties could be shown in canine isolated papillary muscle (Hashimoto, Endoh, Tamura & Taira, 1970) or in intact dogs (Giudicelli *et al.*, 1969), cats (Lubawski & Wale, 1969) or rats (Robak & Gryglewski, 1971). In at least 2 studies in guinea-pig isolated atria (Grodzinska & Gryglewski, 1971) and tracheal chain (Moore & O'Donnell, 1970) the observed sympathomimetic effects of pindolol were attributed to an indirect action involving release of endogenous noradrenaline. In our studies also we have been unable to provide any evidence to suggest that pindolol itself possesses partial agonist activity.

On the basis of metabolic considerations alone there would be no justification in drawing any parallel between these results in DS-rats and observations on pindolol's antihypertensive action in man. For instance in the rat, the biological half-life for the disappearance of pindolol from plasma, following oral or intravenous administration, is reported to be approximately 30 min (Pacha, 1969). Moreover in rats, pindolol is extensively metabolized, the metabolites arising principally from side-chain conjugation with glucuronic acid, hydroxylation or oxidation and conjugation of the indole ring, and oxidative ring scission (Kiechel *et al.*, 1975). In contrast, the biological half-life of pindolol in human plasma, following oral administration, is reported to be approximately 3 h (Pacha, 1969). Furthermore, 40% of an oral dose of pindolol is excreted unchanged in the urine and more than 90% of the metabolites are glucuronides and sulphates (Ohnhaus, Nuesch, Meier & Kalbarer, 1974). Certainly there is no evidence to suggest that pindolol's clinical effectiveness as an antihypertensive drug is in any way attributable to partial agonist activity.

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# FELINE ENDOTOXIN SHOCK: EFFECTS OF METHYLPREDNISOLONE ON KININOGEN-DEPLETION, ON THE PULMONARY CIRCULATION AND ON SURVIVAL

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1 *Escherichia coli* endotoxin, administered intravenously in a dose of 2mg/kg to pentobarbitone-anaesthetized, artificially ventilated cats resulted in pulmonary hypertension, systemic hypotension and an immediate (1–2 min) 30–40% reduction in plasma kininogen, an effect which probably indicates a release of plasma kinins.

2 Methylprednisolone (30 mg/kg), when administered 30 min before endotoxin, did not influence the endotoxin-induced pulmonary hypertension or systemic hypotension but completely prevented the depletion of plasma kininogen.

3 In spontaneously breathing cats, methylprednisolone, administered 30 min *after* endotoxin, caused a rapid repletion of kininogen and prolonged survival (47% at 6 h compared to 10% in the endotoxin-alone animals). Methylprednisolone did not appear to influence lactate production or the hyperventilation observed during the delayed endotoxin shock phase.

4 It is concluded that methylprednisolone does not prevent the release, by endotoxin, of a pulmonary vasoconstrictor prostaglandin, or its effects, but that perhaps by preventing kinin release it may reduce endotoxin-induced capillary leakage.

## Introduction

There is considerable evidence that adrenocortical steroids are of value in the treatment of shock induced by bacterial endotoxins (Kadowitz & Yard, 1970; Berry, 1971; Rao & Cavanagh, 1971; Nicholas & Mela, 1975). The precise mechanisms of this protection are uncertain. One possibility is interference with the release of vasoactive agents by endotoxin or with their effects. These agents include histamine, plasma kinins and prostaglandins.

In a number of species marked pulmonary changes occur early in endotoxin shock. These changes include pulmonary vasoconstriction, increased airways resistance, reduced lung compliance, pulmonary oedema and reduced pulmonary gas exchange leading to an increased alveolar-arterial oxygen tension gradient and a reduced arterial oxygen tension (Kuida, Gilbert, Hinshaw, Brunson & Visscher, 1961, Gemer, Hayes, Ishikawa, Cuevas & Hirsch, 1973; Parratt, 1973; Parratt & Sturgess, 1976). Similar changes occur in patients with septic shock (Wilson, 1972a; Milligan, MacDonald, Mellon & Ledingham, 1974; Sykes, 1976). Recently it has been suggested that, at least in the cat, these pulmonary changes result partly from the liberation by endotoxin of a pulmonary vasoconstrictor, bronchoconstrictor prostaglandin (Parratt & Sturgess, 1975a; 1977).

In view of Wilson's work (1972b) on the effect of methylprednisolone in preventing cellular damage in the post-traumatic lung, we investigated the possibility that methylprednisolone might exert these beneficial pulmonary effects by interfering with the release by endotoxin of this prostaglandin, or with its action on the pulmonary circulation. This study also includes an account of the effects of methylprednisolone on the release of a plasma kinin, another highly vasoactive agent released in the early stages of endotoxin shock (Miller, Reichgott & Melmon, 1973). A preliminary account of some of this work was presented to a meeting of the Physiological Society (Al-Kaisi, Parratt, Siddiqui & Zeitlin, 1976).

## Methods

Cats of either sex were anaesthetized with sodium pentobarbitone (30 mg/kg, i.p.), the chest was opened and the animals ventilated with room air using a Palmer positive-pressure pump (rate, 20/min; stroke volume, 40–60 ml). Mid-oesophageal temperature was recorded with direct reading thermocouples (Ellab, Copenhagen). Systemic (carotid) arterial blood pressure and pulmonary artery pressure were measured as previously described (Parratt, 1973) and recorded, together with the electrocardiogram, on an eight-channel ink-jet writing recorder (Elema-Schönander mingograph 81).

Blood samples (0.6 ml) were taken, without exposure to air, from the catheter in the carotid artery and analysed for $O_2$ and $CO_2$ tensions, and for pH, using appropriately calibrated electrode systems (Parratt, 1973). Blood gas tensions, and pH, were corrected for any temperature difference between the electrode system (usually 37.3°C) and the animals mid-oesophageal temperature by means of a blood-gas calculator (Severinghaus, 1966). Arterial blood samples (2 ml) were also taken for the extraction of kininogen by the method of Brocklehurst & Zeitlin (1967). The samples were immediately added to 6 ml of pre-cooled (4°C) 90% ethanol. The denatured plasma protein was suspended in 6 ml 0.9% w/v NaCl solution (saline) for homogenization and 0.2 ml of this homogenate was incubated with 200 µg of crystalline trypsin for 30 minutes. The reaction was stopped by heating in boiling water for 10 min and the liberated kinin assayed on the stilboestrol-treated rat isolated uterus, bathed in de Jalon's solution containing 1 µg/ml atropine sulphate, in a 5 ml organ bath. The bath temperature was 33°C and the solution was gassed with 95% $O_2$ and 5% $CO_2$. The kinin concentrations of the samples were assayed by close bracketing between doses (usually 1.0 and 1.2 ng) of purified bradykinin (Sandoz, Basle). Total plasma protein was estimated by the Biurett method using a Boehringer test kit. Each time blood was removed from the cat it was replaced by an equal volume of dextrose-saline solution.

For studies designed to determine the effects of methylprednisolone on the secondary, delayed shock phase (Parratt & Sturgess, 1975b) cats were anaesthetized as described above but were not subjected to thoracotomy. Only carotid arterial and right atrial pressures, temperature and the electrocardiogram were recorded in these spontaneously breathing animals. Arterial lactate was measured as described previously (Parratt & Sturgess, 1975b).

All the cats received *Escherichia coli* endotoxin (Difco Laboratories, 055:B5) suspended in saline and administered slowly, by intravenous injection, in a dose of 2 mg/kg. Methylprednisolone sodium succinate (Solumedrone, Upjohn Laboratories, Sussex) was injected intravenously in a dose of 30 mg/kg.

## Results

### *The effect of endotoxin on plasma kininogen and on systemic and pulmonary arterial pressures*

Two groups of cats were studied (Table 1); in the first, blood samples were taken for kininogen analysis before, and 5, 15, 30 and 60 min after, the injection of endotoxin. In the second series, samples were taken 1–2 min after endotoxin (i.e. at the height of the pulmonary pressor response) and again after 30 min; the effect of endotoxin on plasma protein was also examined in this second group of cats. They were later used to examine whether methylprednisolone influenced plasma kininogen depletion when administered 30 min after endotoxin. It is clear that the administration of endotoxin resulted in a considerable (30–40%) and immediate (within 1–2 min) depletion of plasma kininogen and that this was unrelated to changes in packed cell volume (Table 1). It was also unrelated, at least initially, to changes in total plasma protein. Thus immediately before endotoxin the plasma protein was 6.26 ± 0.12 g/100 ml plasma; 2 min after endotoxin, when plasma kininogen was already reduced by 30% (Table 1), it was 6.38 ± 0.21 g/100 ml. Thereafter it was decreased to 6.01 ± 0.17 g/100 ml ($P < 0.05$) after 30 minutes.

The immediate haemodynamic responses of anaesthetized, artificially ventilated cats to *E. coli*

**Table 1** Effects of endotoxin on plasma kininogen levels (as µg bradykinin equivalents/ml plasma) and on packed cell volume (%s in brackets) in anaesthetized cats

| | *Pre-endotoxin* | *After endotoxin* | | | | |
|---|---|---|---|---|---|---|
| | | *+1.5 min* | *+5 min* | *+15 min* | *+30 min* | *+1 h* |
| Series 1 ($n = 5$) | 2.75 ± 0.08<br>(33.8 ± 1.6) | | 1.88 ± 0.12*<br>(33.2 ± 1.5) | 1.69 ± 0.09*<br>(32.8 ± 1.3) | 1.41 ± 0.10*<br>(32.2 ± 1.2) | 1.80 ± 0.23**<br>(31.8 ± 1.2) |
| Series 2 ($n = 7$) | 2.58 ± 0.18<br>(35.2 ± 2.1) | 1.8 ± 0.1**<br>(31.7 ± 3.2) | | | 1.54 ± 0.04*<br>(35.3 ± 2.4) | |

Values are mean ± s.e. mean.
* $P < 0.001$; ** $P < 0.01$.

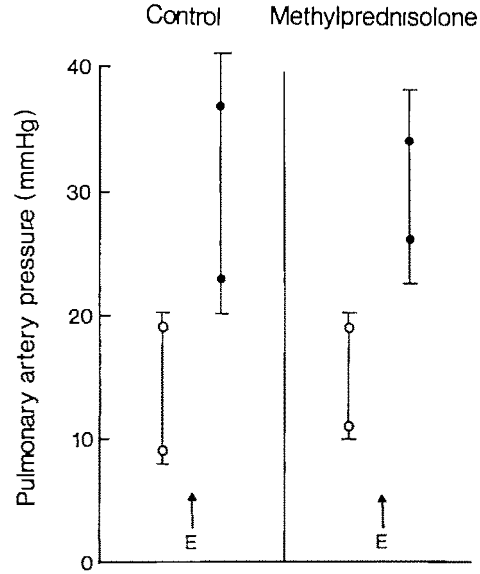

**Figure 1** Effects of endotoxin (E) on pulmonary artery pressure (systolic and diastolic, mmHg) in control cats and in cats pretreated with methylprednisolone (30 mg/kg) 30 min previously. The open circles represent mean values before endotoxin and the closed symbols mean values 3 min after endotoxin The short vertical lines above systolic pressures and below the diastolic pressures represent the s.e. mean; the continuous vertical line joining systolic and diastolic pressures represents the pulmonary pulsatile pressure.

endotoxin have already been documented (Parratt, 1973). In this particular study there was an immediate increase in pulmonary artery pressure, bradycardia and a marked, though transient, reduction in systemic arterial pressure (Table 2).

*Direct haemodynamic effects of methylprednisolone*

Methylprednisolone was administered intravenously, in a dose of 30 mg/kg to a group of 7 anaesthetized cats. There was a moderate (20 mmHg) but transient, reduction in systemic arterial pressure within 1–2 min of the injection (from 113 ± 6 mmHg systolic and 89 ± 9 mmHg diastolic before the administration of methylprednisolone to 93 ± 5 and 62 ± 9 mmHg respectively after 1–2 min; $P < 0.01$). Systemic arterial pressure returned to control levels within 5–10 minutes. There were no significant effects on heart rate (208 ± 11 beats/min before and 204 ± 13 beats/min after the injection), pulmonary arterial pressure (20 ± 11 mmHg systolic and 11 ± 1 mmHg diastolic before methylprednisolone and 18 ± 1 mmHg systolic and 11 ± 1 mmHg diastolic 1–2 min after the injection), plasma kininogen (2.66 ± 0.10 µg bradykinin equivalent/ml plasma before and 2.66 ± 0.11 µg/ml 20 min afterwards) or on packed cell volume (33.1 ± 2.1% before and 33.1 ± 1.8% after).

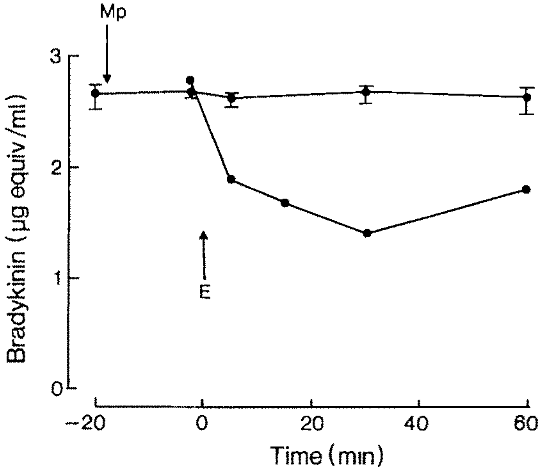

**Figure 2** The effect of pretreatment with methylprednisolone (Mp, 30 mg/kg) on endotoxin-induced plasma kininogen depletion. In cats not treated with methylprednisolone (lower curve) there is a marked depletion of kininogen after the administration of endotoxin (at E). No such depletion is observed (top curve) in animals pretreated with methylprednisolone. The detailed values for the endotoxin-alone experiments are given in Table 1 (series 1).

*The effects of pretreatment with methylprednisolone on the acute haemodynamic response to endotoxin and on endotoxin-induced plasma kininogen depletion*

The haemodynamic effects of endotoxin when given to cats 30 min after pre-treatment with methylprednisolone (30 mg/kg) are summarized in Table 2. There was no modification either of the immediate systemic hypotension, or of the marked pulmonary pressor response (Figure 1). However, endotoxin-induced depletion of plasma kininogen was not observed in these cats (Figure 2), neither was there any change in packed cell volume (33.14 ± 1.75% before endotoxin and 29.7 ± 2.1%, 30.5 ± 2.0% and 28 ± 2.0%, 5, 30 and 60 min after endotoxin).

*Effects of methylprednisolone, administered after endotoxin, on kininogen depletion, on the delayed shock phase and on survival*

It was of interest to determine whether, as well as preventing endotoxin-induced kininogen depletion when given before endotoxin, methylprednisolone modified kininogen depletion when administered after

**Table 2** Haemodynamic effects of endotoxin in open-chest cats pretreated with either saline (control; C) or methylprednisolone (Mp) 30 mg/kg given 30 min previously

| | | *Carotid blood pressure (mmHg)* | | | | *Pulmonary artery pressure (mmHg)* | | | | *Heart rate (beats/min)* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Systolic* | | *Diastolic* | | *Systolic* | | *Diastolic* | | | |
| | | *C* | *Mp* | *C* | *Mp* | *C* | *Mp* | *C* | *Mp* | *C* | *Mp* |
| Pre-endotoxin | | 110±5 | 121±11 | 83±5 | 85±10 | 19±1 | 18±1 | 9±1 | 11±1 | 196±8 | 203±16 |
| Post-endotoxin | +1 to 2 min | 75±9* | 66±13* | 50±8* | 37±9* | 34±3* | 34±4* | 24±2* | 26±3* | 168±10* | 162±20* |
| Post-endotoxin | +30 min | 99±6 | 99±9 | 68±5 | 60±7 | 25±2 | 18±2 | 13±1 | 12±2 | 208±8 | 196±18 |

Values are means ± s.e. mean of 8 to 13 observations.
* $P<0.01$.

**Table 3** Haemodynamic and metabolic effects of endotoxin in spontaneously-breathing anaesthetized cats administered either saline (control, C; $n=10$) or methylprednisolone (Mp; 30 mg/kg; $n=17$) 30 min after endotoxin

| | *Carotid blood pressure (mmHg)* | | | | *Arterial blood pH (units)* | | *Arterial lactate (mg/100 ml)* | | *Survivors (%)* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Systolic* | | *Diastolic* | | | | | | | |
| | *C* | *Mp* | *C* | *Mp* | *C* | *Mp* | *C* | *Mp* | *C* | *Mp* |
| Pre-endotoxin | 134±7 | 128±7 | 104±7 | 102±7 | 7.36±0.02 | 7.38±0.02 | 4.9±1.8 | 4.8±1.8 | — | — |
| Post-endotoxin | | | | | | | | | | |
| +2–3 min | 90±12* | 75±10* | 67±12* | 57±8* | — | — | — | — | 100 | 100 |
| +1 h | 105±7 | 106±6 | 75±8* | 68±5 | 7.25±0.03* | 7.38±0.02 | 23±4* | 17±2* | 100 | 100 |
| +2 h | 119±7 | 84±4 | 82±9 | 68±4 | 7.36±0.03 | 7.41±0.02 | 18±3* | 19±2* | 100 | 100 |
| +3 h | 111±8 | 130±5 | 90±6 | 90±6 | 7.39±0.01 | 7.39±0.02 | 22±5* | 22±3* | 90 | 100 |
| +4 h | 110±11 | 128±4 | 77±14 | 79±8* | 7.32±0.03 | 7.39±0.02 | 23±5* | 22±3* | 70 | 88 |
| +5 h | 88±19* | 95±14 | 62±19 | 79±11 | 7.24±0.14* | 7.43±0.05 | 31±8* | 22±3* | 50 | 77 |
| +6 h | 52 | 110±7 | 36 | 77±11* | 7.41 | 7.42±0.06 | 27.5 | 25±5* | 10 | 47 |

Values are means ± s.e.mean.
* $P<0.01$.

endotoxin. Seven cats (series 2, Table 1) were used for this study; all exhibited a marked pulmonary pressor response to endotoxin and all showed marked depletion of plasma kininogen at 30 min (Table 1). Methylprednisolone was given 31 min after the administration of endotoxin and, within a further 30 min, the plasma kininogen had returned to normal levels (from 1.54 ± 0.04 μg bradykinin equivalents/ml plasma immediately before methylprednisolone to 2.49 ± 0.19 μg/ml, a value not significantly different from the pre-endotoxin level of 2.58 ± 0.18 μg/ml). Methylprednisolone, administered at this time after endotoxin, did not affect packed cell volume (35.3 ± 2.4 to 36.7 ± 2.5%) or plasma protein (6.01 ± 0.17 to 5.81 ± 0.16 mg/100 ml plasma) nor did it modify the endotoxin-induced hypotension. In fact there was some evidence in the group of spontaneously breathing cats (see below) that methylprednisolone administered 30 min after endotoxin delayed the recovery of systemic pressure (values at 1 h and 2 h in Table 3).

A more detailed study of the effect of methylprednisolone on the delayed endotoxin-shock phase was examined in 17 spontaneously breathing cats. The drug was again administered in a dose of 30 mg/kg, 30 min after endotoxin and effects on blood pressure, heart rate, arterial blood gases, pH and lactate were compared with those obtained in a group of 10 cats given only endotoxin. The results are summarized in Table 3. The greatest effect was the increased survival in the cats administered methylprednisolone; 8 out of 17 (47%) were alive at 6 h after endotoxin compared with only 1 (10%) of the group administered endotoxin alone. Systemic arterial pressure was reasonably well maintained in the survivors (110 ± 7 mmHg systolic and 77 ± 11 mmHg diastolic at 6 h) although there was no difference between the two groups with regard to lactate production (Table 3). Arterial blood pH was well maintained, especially in the methylprednisolone group, because of the substantial degree of hyperventilation. For example, the arterial $P\text{CO}_2$ before endotoxin in this group was 34 ± 1 mmHg (within the normal range for spontaneously breathing cats; Parratt, 1973); after 4 h of shock it was reduced to 25 ± 4 mmHg ($P < 0.01$) and after 5 h to 21 ± 3 mmHg ($P < 0.001$). In one of the cats it was only 13 mmHg at 6 h (from the control level of 37 mmHg). This hyperventilation resulted in a gradually increasing arterial $P\text{O}_2$ (from 99 ± 3 mmHg before, to 107 ± 5, 111 ± 7 and 116 ± 7 mmHg, 4, 5 and 6 h after endotoxin). Similar changes in arterial $P\text{CO}_2$ and $P\text{O}_2$ were also observed in the cats given only endotoxin.

## Discussion

Our initial hypothesis that methylprednisolone might prevent the pulmonary effects of bacterial endotoxin by inhibiting the release, or actions, of the vasoconstrictor, bronchoconstrictor, prostaglandin was not supported by these studies. The pulmonary hypertension and oedema that result from the administration of endotoxin in anaesthetized cats were unaffected by methylprednisolone (Figure 1). However, there were three positive effects of methylprednisolone, prevention of the kininogen depletion that resulted from endotoxin administration (Table 1), a rapid repletion of kininogen when given after endotoxin, and increased survival when assessed at 6 hours.

It is clear from these experiments that, in the cat, as in subhuman primates (Herman, Oshima & Erdös, 1974), endotoxin decreases the concentration of plasma kininogen. Such a depletion of kininogen can be equated with the liberation of plasma kinins since there were no significant changes in packed cell volume or in total plasma protein. Other studies have demonstrated that there is a significant correlation between kinin production and kininogen depletion when endotoxin is added to human plasma (Nies & Melmon, 1971) and that free kinins appear in the blood after the administration of endotoxin to human subjects (Kimball, Melmon & Wolff, 1972) and to monkeys (Nies, Forsyth, Williams & Melmon, 1968). We made no attempt to demonstrate a release of free kinins since recoveries of added bradykinin to cat blood were too variable. Recovery of added bradykinin to blood, and other fluids, obtained from other species have been perfectly satisfactory (Brocklehurst & Zeitlin, 1967).

The mechanism by which methylprednisolone prevents kininogen depletion by endotoxin is obscure and any explanation would need to take into account the rapid repletion of the kinin precursor when the drug is administered early in endotoxin shock. In monkeys, the administration of endotoxin leads to activation of plasma kallikrein and ultimately, to a reduction in its concentration in the plasma; a reduction in plasma kallikrein was not seen in monkeys treated with methylprednisolone (Herman *et al.*, 1974). This could indicate that methylprednisolone prevents an effect of endotoxin on complement, on Hageman factor, on plasminogen, or more likely, that it interferes with the interrelationship between endotoxin and granulocytic leucocytes (Miller, Webster & Melmon, 1975), perhaps by stabilizing the cell membrane. There is evidence for at least one of these possibilities; in rats, endotoxin results in a 28% reduction in Hageman factor, an effect completely prevented by pretreatment with glucosteroids (Latour & Leger, 1975).

Whether the effects of methylprednisolone on the kallikrein-kinin system (Table 1) and on survival (Table 3) are related is uncertain. There certainly appears to be no relationship between kinin production and the early (0–30 min) hypotensive phase of endotoxin shock in the cat. The reasons for this are twofold: (i) Despite the failure of endotoxin to reduce plasma kininogen in cats pretreated with methylprednisolone, the initial reduction in blood pressure following endotoxin administration was still evident (Table 2). (ii) Repletion of kininogen by methylprednisolone, when administered 30 min after endotoxin, did not result in a faster recovery from systemic hypotension; in fact recovery appeared to be delayed (Table 3). However, it is conceivable that by preventing the release of free kinins from plasma kininogen, methylprednisolone might prevent the egress of fluid from the circulation into the tissues, an effect which would presumably result from the marked action of the plasma kinins on capillary permeability.

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# REVERSAL OF PROSTAGLANDIN $E_2$ EFFECT ON ADRENAL CATECHOLAMINE RELEASE AFTER HYPOPHYSECTOMY

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Prostaglandin $E_2$ induced increased catecholamine release *in vitro* from adrenals of hypophysectomized rats, while in adrenals of intact rats catecholamine release was suppressed by prostaglandin $E_2$.

**Introduction** Catecholamine release from adrenergic nerve terminals has been shown to be modulated by $\alpha$-adrenoceptor agonists as well as by prostaglandins, mainly prostaglandin E (Hedqvist, 1970; Langer, 1974; Starke & Endo, 1976). We have reported that a similar modulation can be demonstrated in the adrenal medulla, i.e. inhibition of catecholamine release, in an *in vitro* incubated adrenal, by the addition of either phenylephrine or prostaglandin $E_2$ (Boonyaviroj & Gutman, 1975; Gutman & Boonyaviroj, 1977). These effects were found both in rat and in human adrenal medulla slices, incubated *in vitro* (Boonyaviroj & Gutman, 1977). However, when catecholamine release from slices of human phaeochromocytoma, incubated *in vitro*, was studied, prostaglandin $E_2$ consistently caused augmentation of catecholamine release rather than inhibition (Gutman & Boonyaviroj, 1976). The opposite effect of prostaglandin E on catecholamine release from human adrenal medulla and from phaeochromocytoma was also observed when both tissues were obtained from the same patient (Gutman & Boonyaviroj, 1976). This 'reversal' of prostaglandin effect therefore required elucidation.

Phaeochromocytoma cells differ in two parameters from normal adrenal medulla cells: they are not innervated, and their blood supply does not derive from the adrenal cortex (Winkler & Smith, 1972).

Therefore, we set out to mimic the lack of adrenal cortical blood supply in phaeochromocytoma by reducing the corticosteroid supply to the normal adrenal medulla. This can be achieved by hypophysectomy, which abolishes adrenocorticotrophic hormone (ACTH) secretion and, thereby, reduces corticosteroid release from the adrenal cortex. The effect of prostaglandin $E_2$ on catecholamine release from adrenals of hypophysectomized rats was therefore studied.

**Methods** Male rats of the Hebrew University strain, weight 180–200 g, were used throughout. Hypophysectomy was carried out under anaesthesia with ether-oxygen mixture, through a transpharyngeal approach. A 1 mm hole was drilled into the basophenoid bone and the hypophysis was aspirated by suction. Postoperatively the rats were maintained on Purina-chow, and the drinking fluid contained 0.45% NaCl and 2.5% glucose. The adrenal glands were removed for incubation *in vitro* 12 days after the operation.

Rats were killed by dislocation of the neck and the adrenal glands were immediately taken out. Incubation of adrenal glands was carried out as previously described (Boonyaviroj & Gutman, 1975; Gutman & Boonyaviroj, 1977). Each gland was cut into two halves and was placed in a 50 ml conical flask containing 10 ml of Locke solution of the following composition (mM): NaCl 154, KCl 5.6, $MgCl_2$ 5, $CaCl_2$ 0.5, $NaHCO_3$ 1.8 and glucose 5. Incubation was carried out at 37°C in a thermostatic bath, with constant shaking, for 10 minutes. At the end of incubation the glands were separated from the incubation medium. Both the glands and the incubation medium were acidified, with $HClO_4$ (final concentration 0.4 M). Catecholamines were adsorbed on alumina columns, eluted with 0.05 M perchloric acid and then passed through Bio-rex 70 columns (Feuerstein, Boonyaviroj & Gutman, 1977). Adrenaline and noradrenaline were assayed by the trihydroxyindole method using an Aminco-Bowman spectrofluorimeter (Feuerstein *et al.*, 1977).

Acetylcholine chloride (ACh) was purchased from Sigma Chemical Co., St. Louis, Mo. and prostaglandin $E_2$ was a generous gift of Dr J. Pike, The Upjohn Co., Kalamazoo, Mi.

**Results** Figure 1 shows that the addition of prostaglandin $E_2$ (at $10^{-7}$ M) to rat adrenal glands incubated *in vitro* caused a substantial inhibition of catecholamine release, as we have previously reported, both for rat and for human adrenal medulla (Boonyaviroj & Gutman, 1977). Furthermore, prostaglandin $E_2$ also inhibited the release of catecholamines caused by ACh. However, when the adrenal glands were obtained from rats after hypophysectomy, the addition of prostaglandin $E_2$ to the incubation medium caused a significant increase of catecholamine release. This corroborates the effect of prostaglandin $E_2$ on catecholamine release from human phaeochromocytoma, as previously reported (Gutman & Boonyaviroj, 1976). When catecholamine

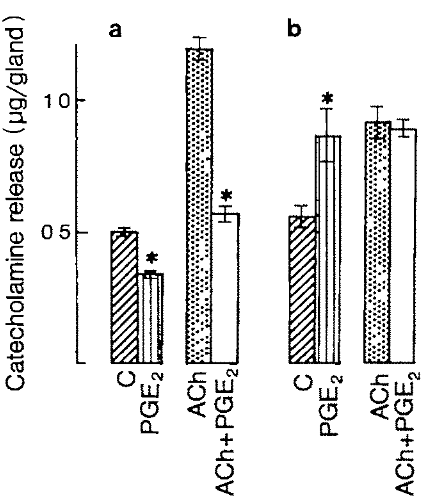

**Figure 1** Effect of prostaglandin E$_2$ on catecholamine release from adrenal glands of control and hypophysectomized rats. C—Incubation of adrenal glands without stimulation; PGE$_2$-incubation of adrenal glands in the presence of prostaglandin E$_2$ $10^{-7}$M. ACh—incubation of adrenal glands in the presence of acetylcholine $10^{-4}$ M. (*a*) Adrenal glands obtained from intact rats. C $n=20$; PGE$_2$ $n=20$; ACh $n=10$; ACh+PGE$_2$ $n=10$. (*b*) Adrenal glands obtained from hypophysectomized rats. C $n=20$; PGE$_2$ $n=22$; ACh $n=21$; ACh+PGE$_2$ $n=23$.

*$P<0.01$ for the difference between adjacent columns.

release from adrenals of hypophysectomized rats was stimulated by ACh, prostaglandin E$_2$ did not inhibit the release (Figure 1). However, there was also no significant increase of catecholamine release above that caused by ACh. This could be due to maximal stimulation of release by ACh, so that no additional release could be observed.

**Discussion** The blood supply of the adrenal medulla derives from the adrenal cortex and, therefore, contains a substantially higher concentration of steroids than blood perfusing other organs. Hypophysectomy abolishes the supply of ACTH to the adrenal cortex and, therefore, reduces the synthesis and release of corticosteroids. Thus, the supply of steroids to the adrenal medulla in hypophysectomized rats is reduced. A similar situation occurs in phaeochromocytoma, where the tumour has a blood supply that does not derive from the adrenal cortex. Under both these conditions, phaeochromocytoma and hypophysectomy, the effect of prostaglandin E$_2$ on catecholamine release was a significant increase, while in adrenal glands from intact rats or normal human adrenal medulla, prostaglandin E$_2$ caused a substantial inhibition of catecholamine release.

The supply of corticosteroids could, therefore, be a determining factor in the type of response to prostaglandin E$_2$, in which case, it would be an interesting example of the modification by steroids of a membrane response. Several possibilities for the mechanism of such a modification may be proposed, e.g. there could be a direct effect of the steroids on membrane composition and/or structure, or the steroids could initiate an intracellular synthetic process which would result in the production of a different receptor incorporated into the membrane. These possibilities are now being studied.

Y.G. is an Established Investigator, Office of Chief Scientist, Israeli Ministry of Health.

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# SIXTH GADDUM MEMORIAL LECTURE

## NATIONAL INSTITUTE FOR MEDICAL RESEARCH, MILL HILL, JANUARY 1977

# *PRESYNAPTIC RECEPTORS AND THEIR ROLE IN THE REGULATION OF TRANSMITTER RELEASE*

S.Z. LANGER[1]
Department of Pharmacology, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS

### Introduction

It is with deep gratitude that I receive the honour of having been selected by the Trustees of the Gaddum Memorial Fund to deliver the Sixth Gaddum Memorial Lecture. Among many other topics, Sir John Gaddum was interested in neurotransmission and he made important contributions in the field of cholinergic (Chang & Gaddum, 1933; Feldberg & Gaddum, 1934) and noradrenergic transmission (Gaddum & Kwiatowski, 1939; Gaddum, Peart & Vogt, 1949; Gaddum, 1950). The work of Sir John Gaddum is also linked to the discovery of other putative neurotransmitters like Substance P and 5-hydroxytryptamine (von Euler & Gaddum, 1931; Gaddum, 1953; Amin, Crawford & Gaddum, 1954). Although I never had a chance to meet Sir John Gaddum personally, as a pharmacologist I always admired his work and his important contributions. In addition, I had the privilege of working for two years with one of Gaddum's collaborators, Dr Marthe Vogt. The time spent with Dr Marthe Vogt in Babraham (1967 and 1968) was a stimulating and rewarding experience. It was during these two years that my interest in noradrenaline release during nerve stimulation started and this has developed further during the last eight years.

The topic of the lecture will be the presence of presynaptic receptors in noradrenergic nerve endings and their role in the regulation of the release of the neurotransmitter upon arrival of nerve impulses.

### Presence of presynaptic $\alpha$-adrenoceptors in noradrenergic nerve endings

Until a few years ago our knowledge of the role of noradrenergic nerve endings in neurotransmission was focused on the synthesis, storage, release and neuronal uptake of the neurotransmitter. During release elicited by the arrival of nerve impulses, the neurotransmitter interacts with specific receptors located in the membrane of the postsynaptic effector cell triggering the typical response of the effector organ: contraction or relaxation of a smooth muscle, positive chronotropic and intropic effects, or secretion of salivary glands. Until recently there were no indications that specific receptors might also be present in the outer surface of the membrane of nerve endings.

In 1957, Brown & Gillespie reported that phenoxybenzamine, an $\alpha$-adrenoceptor blocking agent, increased the overflow of noradrenaline, elicited by nerve stimulation in the perfused cat spleen. These authors postulated that the $\alpha$-adrenoceptor of the effector organ was an important site of loss for the released transmitter. In other words, when the $\alpha$-adrenoceptors of the effector cell were occupied by the blocking agent, the transmitter released during nerve stimulation would not be able to combine with these receptors. Therefore, a larger fraction of the transmitter released by nerve stimulation would be collected in the venous effluent of the perfused spleen, resulting in an increase of the overflow of noradrenaline.

The main sites of loss for noradrenaline released by nerve stimulation are shown schematically in Figure 1: (a) recapture of the released transmitter through neuronal uptake; (b) extraneuronal uptake of noradrenaline; (c) the metabolizing enzymes: monoamine oxidase (coupled with either aldehyde reductase or aldehyde dehydrogenase) and catechol-*O*-methyltransferase, and (d) receptors and other binding sites.

It is clear from Figure 1 that an increase in transmitter overflow can result from the blockade of one or several sites of loss. Alternatively, an increase in transmitter overflow can be due to an actual increase in the release of noradrenaline, whether or not the sites of loss are affected.

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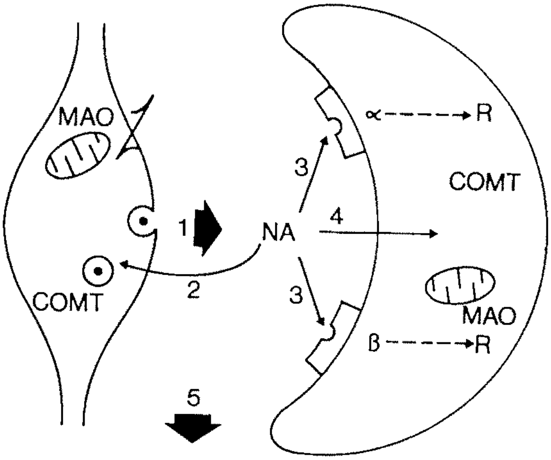

**Figure 1** Main sites of loss for the transmitter released during sympathetic nerve stimulation. (1) Total amount of transmitter released by nerve stimulation. (2) Noradrenaline recaptured by neuronal uptake, subsequently deaminated or stored in vesicles. (3) Fraction of the transmitter released available for activation of the $\alpha$- or $\beta$-postsynaptic receptors, leading to the response (R) of the effector organ. (4) Noradrenaline taken up at extraneuronal sites and subsequently metabolized, predominantly by catechol-*O*-methyltransferase (5) Overflow. noradrenaline collected during and after the period of nerve stimulation. NA: noradrenaline; MAO: monoamine oxidase; COMT catechol-*O*-methyltransferase.

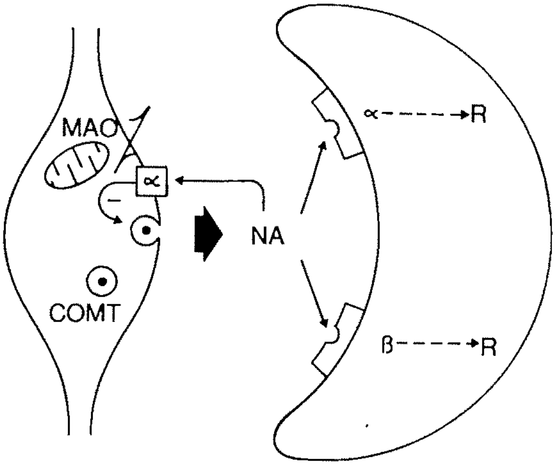

**Figure 2** Schematic representation of the negative feed-back mechanism for noradrenaline released by nerve stimulation, mediated by presynaptic $\alpha$-adrenoceptors. Noradrenaline (NA) released by nerve stimulation once it reaches a threshold concentration in the synaptic cleft activates presynaptic $\alpha$-adrenoceptors leading to inhibition of transmitter release. The presynaptic negative feed-back mechanism is present both in tissues where the response (R) of the effector organ is mediated throuth $\alpha$- or through $\beta$-adrenoceptors. MAO. monoamine oxidase; COMT· catechol-*O*-methyltransferase

The causal relationship between the block of the $\alpha$-adrenoceptors of the effector organ and the increase in transmitter overflow, proposed by Brown & Gillespie (1957) was challenged when it was discovered that phenoxybenzamine was also able to inhibit neuronal uptake (Hertting, 1965; Iversen, 1965) and extraneuronal uptake of noradrenaline (Iversen, 1967; Eisenfeld, Axelrod & Krakoff, 1967; Iversen & Langer, 1969).

However, when a similar degree or even maximal inhibition of neuronal uptake of noradrenaline was obtained with agents which do not block the $\alpha$-adrenoceptors (like cocaine or desipramine) little or no increase in transmitter overflow was observed during nerve stimulation (Blakeley, Brown & Ferry, 1963; Geffen, 1965; Boullin, Costa & Brodie, 1967; Dubocovich & Langer, 1973; Langer & Enero, 1974).

The possible significance of the inhibition by phenoxybenzamine of extraneuronal uptake became apparent after it was reported in studies on transmitter release carried out with [$^3$H]-noradrenaline, that a significant fraction of the $^3$H-transmitter released by nerve stimulation was collected as [$^3$H]-noradrenaline metabolites (Langer, 1970; Langer, Stefano & Enero, 1972; Langer, 1974a; Cubeddu, Barnes, Langer & Weiner, 1974; Langer & Enero, 1974; Luchelli-Fortis & Langer, 1975). Although phenoxybenzamine prevents the metabolism of [$^3$H]-noradrenaline released by nerve stimulation, this effect does not fully account for the increase in transmitter overflow elicited by phenoxybenzamine (Langer, 1970; Langer & Vogt, 1971; Langer, 1974b).

In support of the view that phenoxybenzamine and other $\alpha$-receptor blocking drugs like phentolamine increase the release of the neurotransmitter during nerve stimulation, it was reported that these agents enhance transmitter overflow in concentrations which do not inhibit either neuronal or extraneuronal uptake of noradrenaline (Starke, Montel & Schumann, 1971; Enero, Langer, Rothlin & Stefano, 1972). In addition, it was reported that the release of dopamine-$\beta$-hydroxylase was increased when neurotransmission in the perfused spleen was studied in the presence of phenoxybenzamine or phentolamine (de Potter, Chubb, Put & De Schaepdryver, 1971; Cubeddu *et al.*, 1974). Since dopamine-$\beta$-hydroxylase is a rather large molecule which is not taken up by noradrenergic nerve endings or inactivated by the tissue after its exocytotic release, an increase in overflow of the enzyme does indeed represent an actual increase in release.

Although the increase in transmitter release observed in the presence of $\alpha$-receptor blocking agents was obtained within the range of drug concentrations eliciting $\alpha$-receptor blockade (Enero *et al.*, 1972; Dubocovich & Langer, 1974) a causal relationship between the block of the responses of the effector organ and the increase in transmitter release was excluded because similar results were obtained in guinea-pig isolated atria (Langer, Adler, Enero & Stefano, 1971; McCulloch, Rand & Story, 1972), in the perfused rabbit heart (Starke *et al.*, 1971) and the perfused cat heart (Farah & Langer, 1974) where the adrenoceptors that mediate the response of the effector organ are of the $\beta$-type.

These results led to the hypothesis that $\alpha$-adrenoceptors are present in the outer surface of noradrenergic nerve endings. According to this hypothesis presynaptic $\alpha$-adrenoceptors are involved in the regulation of noradrenaline release through a negative feed-back mechanism mediated by the neurotransmitter itself. As shown schematically in Figure 2, noradrenaline released by nerve stimulation, once it reaches a threshold concentration in the synaptic gap, activates presynaptic $\alpha$-adrenoceptors, triggering a negative feed-back mechanism that inhibits further release of the transmitter (Langer *et al.*, 1971; Farnebo & Hamberger, 1971a; Enero *et al.*, 1972; Starke, 1972a, b; Rand, Story, Allen, Glover & McCulloch, 1973; Langer, 1973; Langer, 1974b).

In support of this hypothesis it has been demonstrated that $\alpha$-adrenoceptor agonists inhibit transmitter release during nerve stimulation (Langer, Enero, Adler-Graschinsky & Stefano, 1972b; Starke, 1972b; Starke, Montel, Gay & Merker, 1974; Starke, Endo & Taube, 1975a; Langer, Dubocovich & Celuch, 1975a). As shown in Table 1, the inhibition of [$^3$H]-noradrenaline release in the perfused cat spleen, obtained by exposure to exogenous noradrenaline in the presence of cocaine, was more pronounced at low frequencies of nerve stimulation. Similar results were obtained in other tissues: the magnitude of the inhibition of [$^3$H]-transmitter release obtained by exposure to $\alpha$-receptor agonists was inversely related to the frequency of nerve stimulation (Starke *et al.*, 1975a).

The frequency-dependence of the inhibition of noradrenaline release obtained with clonidine was also demonstrated under *in vivo* conditions in the rat (Armstrong & Boura, 1973) and in the dog (Scriabine & Stavorski, 1973; Robson & Antonaccio, 1974; Yamaguchi, de Champlain & Nadeau, 1977).

The reduction in transmitter release obtained by exposure to $\alpha$-receptor agonists was equally observed in tissues in which the response of the effector organ is mediated by $\alpha$-receptors (Kirpekar, Furchgott, Wakade & Prat, 1973; Starke *et al.*, 1974; Starke *et al.*, 1975a; Langer *et al.*, 1975a) or by $\beta$-receptors (Starke, 1972b; Langer *et al.*, 1972b; Rand, McCulloch & Story, 1975).

Further support for the hypothesis that noradrenaline release is regulated through a presynaptic negative feed-back mechanism mediated by $\alpha$-adrenoceptors was obtained in experiments in which an increase in noradrenaline release during nerve stimulation was obtained in the presence of $\alpha$-receptor blocking agents. The enhancement in transmitter release obtained by $\alpha$-receptor blocking agents was observed regardless of the $\alpha$- or $\beta$-type of

**Table 1** Influence of the frequency of stimulation on the inhibition by exogenous noradrenaline of $^3$H-transmitter overflow elicited by nerve stimulation in the perfused cat spleen

| *Experimental group* | | | *Fractional release per shock* ($\times 10^{-5}$) | | *Ratio* |
|---|---|---|---|---|---|
| | | n | $S_1$ | $S_2$ | $S_2/S_1$ |
| 1 Hz | Control | 3 | 8.26±3.78 | 8.34±3.31 | 1.11±0.13 |
| | Noradrenaline 0.18 μM | 5 | 6.64±0.85 | 1.92±0.26* | 0.32±0.07** |
| 2 Hz | Control | 3 | 10.22±5.51 | 10.61±6.21 | 1.01±0.08 |
| | Noradrenaline 0.18 μM | 5 | 8.97±0.94 | 5.63±0.62* | 0.64±0.06** |
| 5 Hz | Control | 5 | 8.64±2.53 | 7.07±2.43 | 0.84±0.12 |
| | Noradrenaline 0.18 μM | 4 | 14.14±1.29 | 14.51±1.37 | 1.03±0.03 |

Fractional release per shock: total nCi released per shock divided by the total nCi remaining in the tissue at the onset of nerve stimulation (a total of 300 shocks was delivered at each frequency of stimulation). All experiments were carried out in the presence of cocaine (29 μM); (–)-noradrenaline was added 10 min before the second period of nerve stimulation ($S_2$). Mean values are shown ± s.e. mean. *n*=number of experiments. *$P < 0.025$ when compared against $S_1$ in the same group, **$P < 0.005$ when compared against the ratio of the control group the postsynaptic receptor that mediates the response of the effector organ (Langer, 1970; De Potter *et al.*, 1971; Langer *et al.*, 1971; Farnebo & Hamberger, 1971a; Starke *et al.*, 1971; Enero *et al.*, 1972; Dubocovich & Langer, 1974; Farah & Langer, 1974; Cubeddu *et al.*, 1974; Langer, 1974b; Langer *et al.*, 1975a; Langer, Enero, Adler-Graschinsky, Dubocovich & Celuch, 1975b; Langer, Adler-Graschinsky & Giorgi, 1977).

The negative feed-back mechanism for noradrenaline release during nerve stimulation would be expected to operate most effectively when the transmitter released by nerve impulses reaches a threshold concentration in the synaptic gap. In agreement with this view, it has been shown that when the endogenous noradrenaline stores are depleted either by pretreatment with reserpine or by inhibition of the synthesis of the transmitter by α-methyl-*p*-tyrosine, the effectiveness of phenoxybenzamine in increasing the release of [$^3$H]-noradrenaline and dopamine-β-hydroxylase during nerve stimulation is almost completely lost (Enero & Langer, 1973; Cubeddu & Weiner, 1975). Consequently it appears that when the concentration of released noradrenaline in the synaptic cleft falls below a certain threshold, it fails to trigger the presynaptic negative feed-back mechanism that regulates noradrenaline release.

In support of the view that a threshold concentration of released noradrenaline is required for the activation of the presynaptic negative feed-back mechanism, Rand *et al.* (1975) demonstrated that phenoxybenzamine did not enhance $^3$H-transmitter release from guinea-pig atria elicited by a single pulse, but elicited a 4.5-fold increase in release when a train of 16 pulses was applied.

As already shown for the inhibition of release induced by α-receptor agonists, there was a relationship between the frequency of nerve stimulation and the magnitude of the increase in transmitter release obtained in the presence of α-receptor blocking agents. As the frequency of stimulation is increased, the effectiveness of the α-receptor blocking agents in enhancing noradrenaline release during nerve stimulation is progressively reduced (Brown & Gillespie, 1957; Kirpekar & Cervoni, 1963; Haefely, Hurlimann & Thoenen, 1965; Langer, 1970; Dubocovich & Langer, 1974; Langer *et al.*, 1975a; Dubocovich & Langer, 1976).

An example of the inverse relationship between the frequency of nerve stimulation and the increase in transmitter release induced by α-adrenoceptor blockade in the perfused cat spleen is shown in Table 2. While the increase in transmitter release obtained with phenoxybenzamine at 5 Hz was 6.5-fold, at 30 Hz the increase in release was only 2.4-fold. As shown recently by Dubocovich & Langer (1976) the decreased effectiveness of α-receptor blocking agents in enhancing release of noradrenaline at high frequencies of stimulation cannot be attributed to the attainment of maximal values of transmitter output per shock. It is likely that at high frequencies of nerve stimulation the negative feed-back regulatory mechanism which is mediated by presynaptic α-receptors, does not play an important role in the regulation of transmitter release (Dubocovich & Langer, 1974; Langer *et al.*, 1975a).

Tyramine elicits release of noradrenaline by displacing the transmitter from vesicular binding sites. In contrast to release elicited by potassium or by nerve stimulation, the noradrenaline release induced by tyramine is not calcium-dependent. The presynaptic negative feed-back mechanism that regulates noradrenaline release is operative for release elicited by nerve stimulation or by potassium while this mechanism is not involved in the regulation of transmitter release elicited by tyramine (Starke &

**Table 2** Influence of the frequency of stimulation on the increase in $^3$H-transmitter overflow elicited by phenoxybenzamine in the perfused cat spleen

| | *Experimental group* | n | *Fractional release per shock* ($\times 10^{-5}$) $S_1$ | $S_2$ | *Ratio* $S_2/S_1$ |
|---|---|---|---|---|---|
| 5 Hz | Control | 4 | 10.80 ± 2.86 | 8.68 ± 2.26 | 0.83 ± 0.05 |
| | Phenoxybenzamine | 4 | 8.80 ± 1.14 | 55.89 ± 6.75* | 6.53 ± 0.96** |
| 30 Hz | Control | 4 | 18.00 ± 4.51 | 17.79 ± 4.39 | 1.01 ± 0.17 |
| | Phenoxybenzamine | 4 | 19.64 ± 3.39 | 45.50 ± 5.88* | 2.42 ± 0.26** |

Fractional release per shock: total nCi released per shock divided by the total nCi remaining in the tissue at the onset of stimulation (a total of 300 shocks was delivered at each frequency of stimulation) Phenoxybenzamine 29 μM was added 22 min before $S_2$. Mean values are shown ± s.e. mean. *n*: number of experiments. * $P < 0.01$ when compared against $S_1$ in the same group; ** $P < 0.005$ when compared against the ratio in the control group. (From Dubocovich & Langer, 1976).

Montel, 1973a). It is possible that the presynaptic feed-back mechanism modifies the availability of calcium ions for the release process and that this is the reason why release elicited by tyramine is not influenced by this regulatory mechanism.

It has recently been reported that the inhibition of transmitter release obtained by exposure to exogenous noradrenaline is more pronounced when the calcium concentration in the medium is reduced from 2.6 to 0.25 mM (Langer *et al.,* 1975a). The potentiation of the inhibitory effects of $\alpha$-receptor agonists on neurotransmission by a reduction in the external calcium concentration indicates that activation of presynaptic $\alpha$-receptors may reduce the availability of calcium for the excitation-secretion coupling.

Although both the pre- and postsynaptic $\alpha$-adrenoceptors are stimulated by $\alpha$-receptor agonists and blocked by $\alpha$-receptor antagonists, it appears that the postsynaptic $\alpha$-adrenoceptors that mediate the responses of the effector organ are not identical with the presynaptic $\alpha$-adrenoceptors which regulate the release of noradrenaline during nerve stimulation. When the potency of phenoxybenzamine in blocking the pre- and postsynaptic $\alpha$-receptors was tested in the perfused cat spleen it was found that a significant reduction in responses to nerve stimulation was obtained with low concentrations of the $\alpha$-blocking agent, although transmitter release was not increased under these experimental conditions. As shown in Figure 3, phenoxybenzamine is about 30 times more potent in blocking the postsynaptic $\alpha$-receptors that mediate the responses of the effector organ than it is in blocking the presynaptic $\alpha$-receptors that regulate the release of noradrenaline during nerve stimulation. These results led to the postulate that the pre- and postsynaptic $\alpha$-adrenoceptors are not identical (Dubocovich & Langer, 1974; Langer, 1974b).

These results led to the suggestion that the postsynaptic $\alpha$-adrenoceptors should be referred to as $\alpha_1$ while the presynaptic $\alpha$-adrenoceptors should be referred to as $\alpha_2$ (Langer, 1974b).

In support of this view it was found that in experiments in which the overflow of dopamine-$\beta$-hydroxylase was determined in the perfused cat spleen, phenoxybenzamine was 30 to 100 times more potent in blocking the postsynaptic $\alpha$-receptors than the presynaptic receptors (Cubeddu *et al.,* 1974). On the other hand, these authors found only a very small difference between the potency of phentolamine in blocking the pre- and the postsynaptic $\alpha$-adrenoceptors in the perfused cat spleen.

In further support of the view that the pre- and the postsynaptic $\alpha$-adrenoceptors are not identical, it has recently been shown that the $\alpha$-receptor blocking agent yohimbine was more potent in blocking the presynaptic $\alpha$-receptor than the postsynaptic $\alpha$-receptor in the rabbit main pulmonary artery (Starke, Borowski & Endo, 1975b).

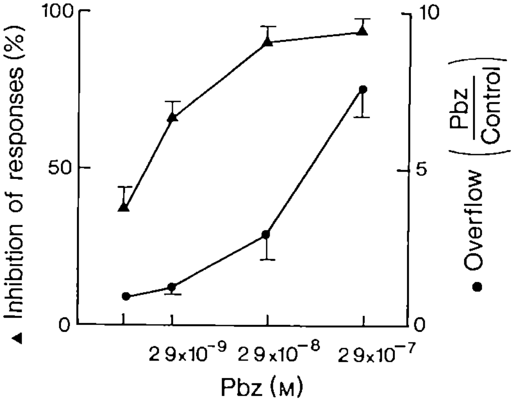

**Figure 3** Differences in potency of phenoxybenzamine in blocking the pre- and postsynaptic adrenoceptors in the perfused cat spleen. Abscissa scale: molar concentration of phenoxybenzamine (Pbz). Left ordinate scale: (▲) percent inhibition of the postsynaptic responses (increase in perfusion pressure induced by nerve stimulation in the presence of different concentrations of phenoxybenzamine). Right ordinate scale· (●) increase in noradrenaline overflow induced by nerve stimulation (5 Hz, during 60 s, supramaximal voltage), expressed as the ratio phenoxybenzamine (Pbz) over control: 1 (no change in transmitter overflow), 5 (five-fold increase in transmitter overflow) Mean values of at least 4 experiments per group are shown. Vertical lines show s.e. means Results from Dubocovich & Langer (1974)

Differences also exist for the $\alpha$-adrenoceptor stimulating agents with regard to their relative potencies on the presynaptic or $\alpha_2$ and the postsynaptic or $\alpha_1$-receptors. Clonidine, $\alpha$-methylnoradrenaline and oxymetazoline are more potent in reducing noradrenaline release during nerve stimulation than in stimulating the postsynaptic $\alpha$-adrenoceptors (Starke *et al.,* 1974; Starke *et al.,* 1975a).

It is noteworthy that the sensitivity of the presynaptic $\alpha$-adrenoceptors can be modified after exposure to a large concentration of an $\alpha$-receptor agonist. In the cat spleen perfused with cocaine, there is a nearly 2-fold increase in transmitter release during nerve stimulation after 60 min of exposure to noradrenaline 0.59 μM (Langer & Dubocovich, 1977). This effect is probably due to a short lasting subsensitivity of the presynaptic $\alpha$-adrenoceptors resulting from the exposure to noradrenaline. In support of this view, it has been found that this phenomenon is not observed in the presence of phentolamine (Langer & Dubocovich, 1977).These results are compatible with the view that chronic stimulation or blockade of presynaptic receptors may lead to changes in their sensitivity to the neurotransmitter, as was already demonstrated for the postsynaptic receptor. Consequently, attention should be drawn to the fact that chronic stimulation or blockade of the presynaptic receptors may lead to changes in their sensitivity which may affect the regulation of neurotransmission.

In spite of the fact that the available evidence indicates that the negative feed-back mechanism for noradrenaline release is of a presynaptic nature, the possibility that a trans-synaptic regulatory mechanism is involved was suggested by several authors (Häggendal, 1970; Farnebo & Malmfors, 1971). Yet there are several pieces of evidence in favour of the presynaptic location of the $\alpha$-adrenoceptor involved in the regulation of noradrenaline release during nerve stimulation.

In recently formed nerve endings from cultured rat superior cervical ganglia, phenoxybenzamine enhances [$^3$H]-noradrenaline release induced by potassium (Vogel, Silbertstein, Berv & Kopin, 1972). Under these experimental conditions, $\alpha$-receptor block enhanced release from nerve endings in the absence of an effector postsynaptic cell.

After short term denervation of the cat nictitating membrane, and at a time when postsynaptic changes in sensitivity have not yet developed (Langer & Trendelenburg, 1966; Langer, Draskoczy & Trendelenburg, 1967; Langer, 1975) there are marked changes in the sensitivity of the presynaptic $\alpha$-adrenoceptors that regulate noradrenaline release during nerve stimulation (Langer & Luchelli-Fortis, 1977). Eighteen hours after surgical denervation of the cat nictitating membrane phentolamine did not increase [$^3$H]-noradrenaline release by nerve stimulation and clonidine was significantly less effective in reducing $^3$H-transmitter release. Yet, 18 h after surgical denervation the ability of phentolamine to block and of clonidine to stimulate the postsynaptic $\alpha$-adrenoceptors did not differ from the controls (Langer & Luchelli-Fortis, 1977). In support of the view that the changes observed in neurotransmission are due to subsensitivity of the presynaptic $\alpha$-adrenoceptors, it was found that 18 and 24 h after denervation the fractional release per stimulus was significantly higher than that obtained in the corresponding controls (Table 3).

Additional evidence in favour of the presynaptic location of the $\alpha$-adrenoceptor involved in the regulation of noradrenaline release was recently obtained in the rat submaxillary gland. These experiments were carried out after atrophy of the secretory cells elicited by duct ligation 15 days before the experiment (Standish & Shafer, 1957). In these atrophied salivary glands, the secretory responses to adrenoceptor or cholinoceptor agonists are abolished at a time when there are no changes in either the cholinergic or the noradrenergic innervation (Filinger, Langer, Perec & Stefano, 1977). Exposure to phentolamine 3.1 µM, increased the release of [$^3$H]-noradrenaline from slices of the normal submaxillary gland elicited by 60 mM potassium 2.97 ± 0.59-fold; under these experimental conditions the increase in release obtained by phentolamine in atrophied salivary glands was 3.65 ± 0.78-fold. Consequently, the $\alpha$-receptor blocking agent was equally effective in increasing transmitter release in normal as well as in atrophied glands. The effect of phentolamine was therefore mediated through a presynaptic mechanism which could be demonstrated whether or not the postsynaptic effector cell was physiologically or pharmacologically involved.

In connection with the physiological significance of the negative feed-back mechanism mediated by presynaptic $\alpha$-adrenoceptors, it should be noted that an enhancement in transmitter release, as observed in the presence of $\alpha$-receptor blocking agents, would be expected to result in the potentiation of the response of the effector organ to nerve stimulation. However, in tissues in which the response of the effector organ is mediated through postsynaptic $\alpha$-adrenoceptors (spleen, nictitating membrane, blood vessels) the responses to nerve stimulation are reduced by the $\alpha$-blocking agents. The only exception found so far concerns yohimbine, which is more potent in blocking

**Table 3** Subsensitivity of the presynaptic $\alpha$-adrenoceptors after short-term surgical denervation of the cat nictitating membrane

| *Experimental group* | n | *Fractional release per shock ($\times 10^{-5}$)* | *Ratio Den/con* |
|---|---|---|---|
| Controls | 9 | 1.01 ± 0.10 | – |
| Denervated 18 h | 16 | 1.83 ± 0.29* | 1.57 ± 0.15** |
| Denervated 24 h | 9 | 2.33 ± 0.39* | 2.42 ± 0.44** |

Fractional release per shock: total nCi released per shock divided by the total nCi remaining in the tissue at the onset of nerve stimulation (10 Hz, during 2 min, supramaximal voltage). Den: denervated. Mean values are shown ± s.e. mean. *n*: number of experiments.

* $P < 0.05$ when compared against the control group; ** $P < 0.025$ when compared against the ratio between right and left normal nictitating membranes. (From Langer & Luchelli-Fortis, 1977).

the presynaptic than the postsynaptic α-adrenoceptors in the rabbit main pulmonary artery (Starke *et al.*, 1975b). Low concentrations of yohimbine are thus able to enhance [$^3$H]-noradrenaline release and potentiate the contractile responses to nerve stimulation (Starke *et al.*, 1975b).

In tissues in which the response of the effector organ is mediated through β-receptors, exposure to α-adrenoceptor blocking agents would be expected to enhance the responses to nerve stimulation because of the increase in transmitter release observed under these experimental conditions. As expected, when the responses to accelerans nerve stimulation at *low* frequencies were determined in guinea-pig atria, phentolamine increased significantly the positive chronotropic responses to nerve stimulation (Langer *et al.*, 1977). Under these experimental conditions, the concentration of the α-receptor blocking agent produced a significant increase in $^3$H-transmitter release during nerve stimulation (Langer *et al.*, 1977).

The significant increase in the positive chronotropic responses to nerve stimulation obtained under experimental conditions in which the release of noradrenaline was enhanced by exposure to phentolamine supports the view that the negative feed-back mechanism mediated by presynaptic α-adrenoceptors plays a major physiological role in noradrenergic neurotransmission. Similar results were obtained under *in vivo* conditions: Lokhandwala & Buckley (1976) demonstrated in the anaesthetized dog that phentolamine potentiated the positive chronotropic responses to cardioaccelerator nerve stimulation.

Neuronal uptake of noradrenaline, by effectively reducing the concentration of the transmitter in the vicinity of the outer surface of the nerve ending (Figure 1) appears to modulate this presynaptic feed-back mechanism, by regulating the fraction of the transmitter released by stimulation which is available to activate the presynaptic α-adrenoceptors (Langer, 1974b). Accordingly, when neuronal uptake is inhibited by cocaine, a higher fraction of the noradrenaline released by nerve stimulation would become available for activation of the presynaptic inhibitory α-adrenoceptors. In support of this view, it was reported that, in the perfused cat spleen, during exposure to concentrations of cocaine ranging from 0.3 to 30 μM, there was no significant increase in the overflow of total tritium elicited by nerve stimulation (Cubeddu *et al.*, 1974). In addition, in the presence of 3 and 30 μM cocaine, there was a concentration-dependent reduction in the overflow of dopamine-β-hydroxylase during nerve stimulation (Cubeddu *et al.*, 1974). These results are compatible with the view that inhibition of neuronal uptake by cocaine leads to a decrease in transmitter output because of enhanced feed-back inhibition by the higher concentration of the transmitter achieved in the vicinity of the nerve ending (Enero *et al.*, 1972; Langer, 1974b; Langer & Enero, 1974).

It is well known that the importance of neuronal uptake in regulating the concentration of noradrenaline in the biophase is inversely related to the neuromuscular distance; in other words, the narrower the neuromuscular gap, the more important is neuronal uptake in the regulation of the concentration of the neurotransmitter in the biophase. The width of the neuromuscular distance would also be expected to modify the presynaptic negative feed-back mechanism because it influences the concentration of the released transmitter achieved in the synaptic cleft. Accordingly, the analysis of results obtained in different tissues with known neuromuscular distances showed that the magnitude of the increase in transmitter release elicited by nerve stimulation in the presence of phentolamine was the more pronounced the smaller the neuromuscular interval of the tissue (Langer *et al.*, 1975b). Consequently, it appears that in organs with narrow neuromuscular gaps the presynaptic feed-back inhibition for noradrenaline release during nerve stimulation plays a more important role as a regulatory mechanism than in tissues with wide synaptic gaps.

It is of interest to note that there is evidence in favour of the presence of neural α-adrenoceptors mediating the negative feed-back control of noradrenaline release in human vasoconstrictor nerves. These experiments were carried out in isolated superfused field stimulated biopsy specimens of human peripheral arteries and veins. An enhancement in $^3$H-transmitter release was demonstrated in the presence of α-receptor blocking agents, as well as inhibition of release induced by exposure to exogenous noradrenaline (Stjärne & Gripe, 1973; Stjärne & Brundin, 1975).

## Evidence for the presence of presynaptic β-adrenoceptors in noradrenergic nerve endings

It has recently been postulated that in addition to the presynaptic negative feed-back mechanism for noradrenaline released by nerve stimulation which is mediated via α-adrenoceptors, a positive feed-back mechanism exists in noradrenergic nerve endings that is triggered through the activation of presynaptic β-adrenoceptors (Langer, Adler-Graschinsky & Enero, 1974; Adler-Graschinsky & Langer, 1975; Langer *et al.*, 1975a; Stjärne & Brundin, 1975; Dahlöf, Ablad, Borg, Ek & Waldeck, 1975; Langer, 1976; Stjärne & Brundin, 1976a; Yamaguchi *et al.*, 1977).

This hypothesis is based on the fact that exposure to low concentrations of isoprenaline enhances the release of noradrenaline during nerve stimulation at low frequencies in several noradrenergically innervated organs: guinea-pig atria, perfused cat

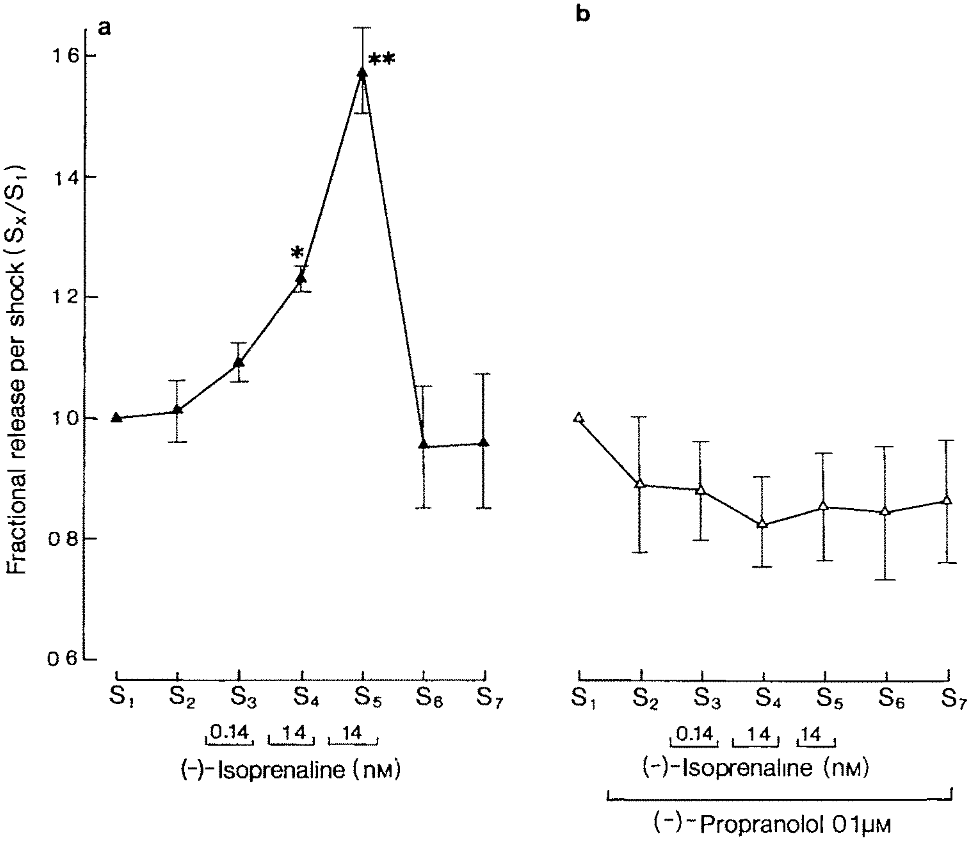

**Figure 4** Increase by isoprenaline of $^3$H-transmitter release elicited by nerve stimulation in the perfused cat spleen. Abscissa scale: $S_1$ to $S_7$ indicate the periods of nerve stimulation (1 Hz, during 120 s, with supramaximal voltage). The interval between each period of nerve stimulation was 20 minutes Ordinate scale: ratio between the fractional release of $^3$H-transmitter per shock obtained during a given period of nerve stimulation ($S_x$) and the first period ($S_1$). (a) (▲) Isoprenaline in the concentration which is indicated was added to the perfusion medium 10 min before $S_3$, $S_4$ and $S_6$; (b) (△) same as in (a) but propranolol 0.1 μM was added 10 min before $S_2$ and was present throughout Note that isoprenaline induced a concentration-dependent increase in $^3$H-transmitter release, which was reversible by washing. The effect of isoprenaline was prevented by exposure to propranolol. Mean values of at least 4 experiments in each group are shown. Vertical lines show s.e. means. (Celuch, Dubocovich & Langer, unpublished)
* $P<0.05$; ** $P<0.005$.

spleen, cat thoracic aorta, cat nictitating membrane, human oviduct and human vasoconstrictor nerves (Langer *et al.*, 1974; Adler-Graschinsky & Langer, 1975; Langer *et al.*, 1975b; Stjärne & Brundin, 1975; Hedqvist & Moawad, 1975; Stjärne & Brundin, 1976a). The increase in transmitter release obtained in the presence of isoprenaline is independent of the $\alpha$ or $\beta$ nature of the response of the effector organ to the transmitter (Langer *et al.*, 1975b).

As shown in Figure 4, the effects of (−)-isoprenaline on transmitter release obtained in the perfused cat spleen were concentration-dependent, and readily reversed by washing. The enhancement in transmitter release obtained with (−)-isoprenaline was stereospecific, since it was not obtained with (+)-isoprenaline (Langer, 1976).

The effects of isoprenaline on transmitter release can be prevented by preincubation with 0.1 μM propranolol (Figure 4). However, acute exposure to propranolol 0.1 μM did not always reduce significantly noradrenaline release during nerve stimulation. Propranolol reduced transmitter release elicited by nerve stimulation in guinea-pig isolated atria (Adler-Graschinsky & Langer, 1975) and in the calf muscle of the cat pretreated with phenoxybenzamine (Dahlöf *et al.*, 1975). Yet, exposure to propranolol 0.1 μM did not reduce $^3$H-transmitter release from human omental arteries and veins (Stjärne & Brundin, 1975).

Considerably higher concentrations of propranolol reduce noradrenaline release during nerve stimulation in several tissues but this effect is related to the local anaesthetic properties of propranolol (Barret & Nunn, 1970; Hughes & Kneen, 1976).

Recently Yamaguchi *et al.* (1977) demonstrated that in the anaesthetized dog an infusion of isoprenaline enhanced the release of noradrenaline elicited by right cardioaccelerator nerve stimulation at low frequencies. These authors also reported that the administration of the β-receptor blocking agent, sotalol reduced significantly the release of noradrenaline at stimulation frequencies between 1 and 5 Hz. Consequently, the physiological role of the presynaptic β-adrenoceptors in noradrenergic neurotransmission has also been demonstrated under *in vivo* conditions.

It is possible that the long-term β-receptor blockade may be more effective in reducing the amount of transmitter released per impulse from noradrenergic nerves. Ljung, Äblad, Dählof, Henning & Hultberg (1975) reported that prolonged administration of propranolol or metoprolol to spontaneously hypertensive rats resulted in a reduction of the responses to postganglionic nerve stimulation in the portal vein preparation without concomitant changes in sensitivity to exogenous noradrenaline. In addition, Lewis (1974) reported that in the pithed rat preparation, chronic, but not acute administration of practolol reduced the pressor responses to preganglionic sympathetic stimulation.

It is noteworthy that Stjärne & Brundin (1975) have shown that isoprenaline increases [$^3$H]-noradrenaline release elicited by field stimulation from strips of human omental arteries and veins. A similar effect was obtained with low concentrations of adrenaline (Stjärne & Brundin, 1975) although a further increase in the concentration of adrenaline led to inhibition of transmitter release due to stimulation of α-presynaptic receptors. Stjärne & Brundin (1975) suggested that the presynaptic β-adrenoceptors can be activated by the levels of circulating catecholamines and may subserve the function of enhancing the secretion of sympathetic transmitter during conditions of increased secretion of adreno-medullary hormone. Isoprenaline also increases the release of [$^3$H]-noradrenaline during nerve stimulation at low frequency in the human oviduct (Hedqvist & Moawad, 1975).

These results are compatible with the existence of β-adrenoceptors in noradrenergic nerve endings. Activation of this mechanism by β-adrenoceptor agonists leads to an increase in transmitter release during nerve stimulation (Figure 5). The increase in transmitter release elicited by β-adrenoceptor activation is more pronounced at low frequencies of nerve stimulation (Langer *et al.*, 1975b).

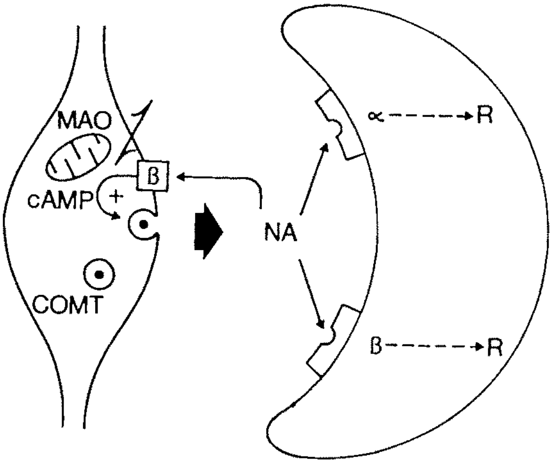

**Figure 5** Schematic representation of the positive feed-back mechanism for noradrenaline released by nerve stimulation, mediated by presynaptic β-adrenoceptors Noradrenaline (NA) released by low frequencies of nerve stimulation activates presynaptic β-adrenoceptors, leading to an increase in transmitter release. This effect appears to be mediated through an increase in the levels of cyclic AMP (cAMP) in noradrenergic nerve endings The presynaptic positive feed-back mechanism is present both in tissues where the response (R) of the effector organ is mediated through α- or through β-adrenoceptors. MAO· monoamine oxidase; COMT· catechol-*O*-methyltransferase.

The presynaptic β-adrenoceptors appear to mediate a positive feed-back mechanism for noradrenaline released at low frequencies of nerve stimulation (Figure 5). The transmitter released at low frequencies would then facilitate its own release through the activation of presynaptic β-adrenoceptors.

The $\beta_1$ or $\beta_2$ nature of the presynaptic β-adrenoceptors is still somewhat controversial. According to Dahlöf *et al.* (1975) the presynaptic receptors are of the $\beta_1$-type because they are blocked by metoprolol, a selective $\beta_1$-receptor blocking agent. Yet, a recent publication by Stjärne & Brundin (1976a) suggests that the presynaptic β-adrenoceptors involved in the facilitation of noradrenaline release are of the $\beta_2$-type because terbutaline and salbutamol enhanced transmitter release, while a $\beta_1$-agonist, H 110/38, was without effect.

Since the results of Dahlöf *et al.* (1975) were obtained in the rat portal vein while those of Stjärne & Brundin (1976a) were obtained with human omental arteries and veins, it is possible that the different results reflect species and tissue differences.

The facilitation of transmitter release triggered by the activation of presynaptic β-receptors may be mediated through an increase in the levels of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) in noradrenergic nerve endings (Figure 5). Wooten, Thoa, Kopin & Axelrod (1973) reported that dibutyryl cyclic AMP and theophylline increase the release of noradrenaline and of dopamine-β-hydroxylase elicited by nerve stimulation in the guinea-pig vas deferens. Papaverine and other phosphodiesterase inhibitors enhance noradrenaline release during nerve stimulation (Langer *et al.*, 1975b; Cubeddu, Barnes & Weiner, 1975). In the perfused cat spleen several cyclic nucleotide analogues enhance both noradrenaline and dopamine-β-hydroxylase release during nerve stimulation (Cubeddu *et al.*, 1975).

Papaverine produces a shift to the left in the concentration-effect curve for (−)-isoprenaline on transmitter release in the perfused cat spleen (Langer, 1976). In addition, the effect of papaverine on transmitter release is significantly reduced by exposure to 0.1 μM propranolol.

It is of interest to note that in the adrenal medulla, Serck-Hanssen (1974) postulated a β-adrenergic system confined to the adrenaline storing cell, which enhances release of the catecholamine and which appears to be mediated by an increase in the cellular concentration of cyclic AMP.

Recently, Roth, Morgenroth & Salzman (1975) have postulated that adenylate cyclase present in noradrenergic nerve endings is involved through increased cyclic AMP formation in the activation of tyrosine hydroxylase as a result of nerve stimulation. At present it is not yet clear as to whether a common presynaptic site of action is involved in the facilitation of noradrenaline release mediated by presynaptic β-adrenoceptors and the increase in tyrosine hydroxylase activity that occurs during and after sympathetic nerve stimulation. Both mechanisms appear to be mediated through an increase in cyclic AMP levels in nerve endings.

In rat striatal slices labelled with [$^3$H]-dopamine, dibutyryl cyclic AMP produces a concentration-dependent increase in [$^3$H]-dopamine release elicited by electrical stimulation (Westfall, Kitay & Wahl, 1976). These results add further support to the view that cyclic AMP may play a role in monoaminergic neurotransmission, acting presynaptically to increase transmitter release.

Recently, Stjärne & Brundin (1976b) demonstrated that isoprenaline enhanced [$^3$H]-noradrenaline release elicited by nerve stimulation of human omental blood vessels even after the local production of prostaglandins was blocked by the addition of 5,8,11,14-eicosatetranoic acid (ETA).

### Working hypothesis for the participation of the presynaptic α- and β-adrenoceptors in the regulation of transmitter release during nerve stimulation

Two presynaptic mechanisms appear to be involved in the autoregulation of noradrenaline release during nerve stimulation. The first one, mediated by β-adrenoceptors, would be activated by low concentrations of noradrenaline (i.e. in the range of low frequencies of nerve stimulation) leading to an increase in transmitter release (Figure 6). The second one, mediated through α-adrenoceptors, is triggered when higher concentrations of the transmitter are reached in the synaptic cleft, leading to inhibition of transmitter release.

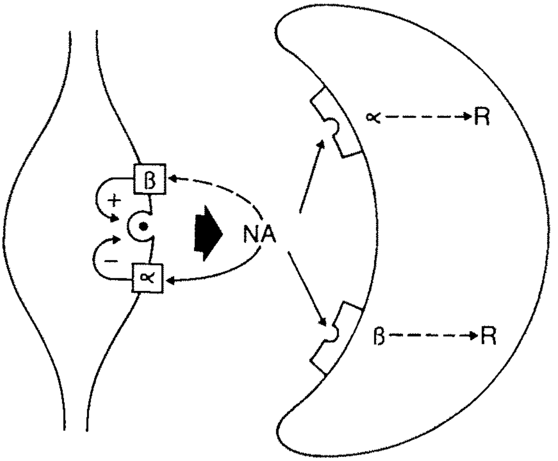

**Figure 6** Role of the presynaptic α- and β-adrenoceptors in the regulation of noradrenaline release during nerve stimulation. During noradrenaline (NA) release at low frequencies of nerve stimulation (when the concentration of the released transmitter in the synaptic cleft is rather low) the positive feed-back mechanism mediated by presynaptic β-adrenoceptors is activated leading to an increase in transmitter release. As the concentration of released noradrenaline increases, a threshold is reached at which the negative feed-back mechanism mediated by presynaptic α-adrenoceptors is triggered, leading to inhibition of transmitter release. Both presynaptic feed-back mechanisms are present in nerves, irrespective of the α or β nature of the receptors that mediate the response (R) of the effector organ.

The positive feed-back mechanism which facilitates transmitter release appears to be mediated through an increase in the cyclic AMP levels in noradrenergic nerve endings. On the other hand, the negative feed-back mechanism which leads to inhibition of noradrenaline release operates by restricting the calcium available for the excitation-secretion coupling.

In support of this working hypothesis, it is of interest to note that, in most tissues, the concentrations of noradrenaline required for stimulation of postsynaptic α-adrenoceptors are about 100 times higher than those necessary to stimulate the postsynaptic β-receptors (Adler-Graschinsky & Langer, 1975; Langer *et al.*, 1975b). If the relative affinities of noradrenaline for the postsynaptic α- and β-adrenoceptors can be extrapolated to the presynaptic receptors, this difference in potency would be compatible with the present hypothesis: low concentrations of released noradrenaline activating presynaptic β-receptors while the presynaptic α-adrenoceptors are activated when a higher, threshold concentration of the transmitter is achieved in the synaptic cleft (Figure 6).

In view of the fact that the most pronounced increases in transmitter release are obtained when the presynaptic α-adrenoceptors are blocked by drugs, it follows that the major regulatory mechanism for noradrenaline release by nerve stimulation under physiological conditions is mediated by presynaptic α-adrenoceptors.

### Other types of presynaptic receptors in noradrenergic nerve endings

In addition to the presynaptic α- and β-adrenoceptors, a variety of presynaptic receptor sites have been described in noradrenergic nerve endings. As shown schematically in Figure 7, the following presynaptic receptors have been postulated: (a) muscarinic inhibitory cholinoceptors (Loffelholz and Muscholl, 1969; Steinsland, Furchgott & Kirpekar, 1973; Langer, Enero, Adler-Graschinsky, Dubocovich & Giorgi, 1976); (b) dopamine inhibitory receptors (Langer, 1973; McCulloch, Rand & Story, 1973; Enero & Langer, 1975; Long, Heintz, Cannon & Kim, 1975); (c) opiate inhibitory receptors, which are activated by morphine and also by the naturally occurring pentapeptides met and leu-enkephalin (Hughes, Kosterlitz & Leslie, 1975; Dubocovich & Langer, unpublished observations); (d) prostaglandin inhibitory receptors, which are activated by prostaglandin $E_1$ and $E_2$ (Hedqvist, 1970; Stjärne, 1973; Dubocovich & Langer, 1975; Hedqvist, 1976); (e) adenosine inhibitory receptors (Hedqvist & Fredholm, 1976); (f) angiotensin II facilitatory receptors (Starke, 1970, 1971; Hughes & Roth, 1971) and (g) nicotinic facilitatory receptors (Lindmar, Löffelholz & Muscholl, 1968; Löffelholz, 1970).

In connection with the multiplicity of presynaptic receptor sites (Figure 7) it should be noted that some of these receptors are not present in all noradrenergic nerve endings of the peripheral nervous system. The presynaptic opiate receptors are not present in the rabbit heart (Montel & Starke, 1973), or in the guinea-pig heart (Dubocovich & Langer, unpublished observations). Presynaptic angiotensin receptors are not present in the perfused cat spleen (Hertting & Suko, 1966; Langer & Enero, unpublished observations) and prostaglandin receptors of the E series are not present in the cat nictitating membrane (Enero & Langer, unpublished observations).

Except for the α- and β-presynaptic adrenoceptors, it appears that the other presynaptic receptors do not play a physiological role in noradrenergic neurotransmission and consequently are of pharmacological rather than physiological importance. However, as discussed below, these receptors can be acted upon by agonists or their analogues to modify sympathetic neurotransmission in the peripheral nervous system.

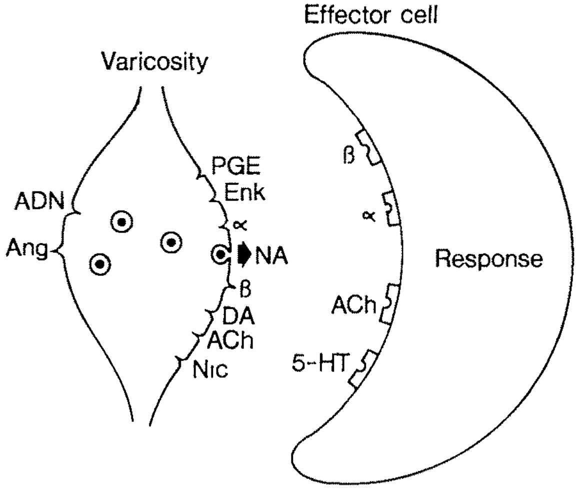

**Figure 7** Schematic representation of the pre- and postsynaptic receptors in a noradrenergic neuroeffector junction in the peripheral nervous system. Noradrenergic varicosity. (1) PGE: prostaglandins of the E series, inhibition of noradrenaline (NA) release; (2) Enk: enkephalin opiate receptors: inhibition of NA release; (3) α-adrenoceptors: inhibition of noradrenaline release, (4) β-adrenoceptors, enhancement of noradrenaline release; (5) DA: dopamine receptors, inhibition of noradrenaline release; (6) ACh: muscarinic cholinoceptors, inhibition of noradrenaline release; (7) Nic: nicotinic receptors, enhancement of noradrenaline release; (8) ADN: adenosine receptors, inhibition of noradrenaline release; (9) Ang: angiotensin II receptors, enhancement of noradrenaline release.
Effector cell Smooth muscle postsynaptic receptors. (1) β-adrenoceptors: relaxation; (2) α-adrenoceptors: contraction, (3) ACh: muscarinic cholinoceptors, contraction; (4) 5-HT: 5-hydroxytryptamine receptors, contraction. The smooth muscle of the cat nictitating membrane was used as an example

The experimental evidence accumulated during recent years indicates that in addition to the multiple receptor sites in the postsynaptic effector cell there are also multiple receptor sites in the presynaptic membrane of nerve endings (Figure 7). Further research in the field of presynaptic receptors may clarify their physiological role and eventually open up new possibilities in therapeutics.

It has been suggested that activation of presynaptic α-adrenoceptors in the peripheral sympathetic system contributes to the antihypertensive effects of clonidine (Armstrong & Boura, 1973; Starke *et al.*, 1974; Langer *et al.*, 1975b; Langer, 1976).

More recently, it has been postulated that block of the presynaptic $\beta$-adrenoceptors in the periphery contributes significantly to the antihypertensive effects of $\beta$-receptor blocking agents (Langer *et al.*, 1975b; Adler-Graschinsky & Langer, 1975; Ljung *et al.*, 1975; Langer, 1976; Yamaguchi *et al.*, 1977).

## Evidence for the presence of presynaptic receptors in the central nervous system

Results obtained on [$^3$H]-noradrenaline release in several areas of the central nervous system indicate that a similar negative feed-back mechanism mediated by presynaptic $\alpha$-adrenoceptors is operative in noradrenergic nerve endings in the brain (Farnebo & Hamberger, 1971b; Starke & Montel, 1973b; Dismukes & Mulder, 1976). These conclusions are based on the observations that $\alpha$-adrenoceptor agonists decrease while $\alpha$-adrenoceptor blocking agents increase the release of [$^3$H]-noradrenaline induced by electrical field stimulation or by potassium in brain slices.

It has been suggested that activation by clonidine of *presynaptic* $\alpha$-adrenoceptors in the central nervous system is causally related to its antihypertensive effects (Starke & Altman, 1973). Yet, Haeusler (1974) and Kobinger & Pichler (1976) demonstrated that clonidine and other imidazolines exert their sympatho-inhibitory effects and thus hypotension and bradycardia, by stimulation of *postsynaptic* rather than *presynaptic* $\alpha$-adrenoceptors in the central nervous system.

The $\alpha$-adrenoceptors which mediate the decrease in central turnover of noradrenaline have been shown to differ from the central postsynaptic $\alpha$-receptors and are probably presynaptic (Anden, Grabowska & Strombom, 1976). In addition, it appears that presynaptic $\alpha$-adrenoceptors in the central nervous system are involved in the analgesia and locomotor depression caused by clonidine (Paalzow & Paalzow, 1976; Strömbom, 1976).

The presence of presynaptic opiate receptors has been demonstrated in noradrenergic nerve endings of the rat cerebral cortex (Montel, Starke & Weber, 1974), although the physiological relevance of these presynaptic receptors and the role of the endogenous enkephalins remains to be clarified.

Presynaptic mechanisms in central dopaminergic neurones have also been described. These presynaptic dopaminergic receptors appear to be involved in the regulation of dopamine synthesis and release (Farnebo & Hamberger, 1971b; Carlsson, 1975; Roth, Walters, Murrin & Morgenroth, 1975; Iversen, Rogawski & Miller, 1976). Yet, the role of the presynaptic dopamine receptors in the regulation of transmitter release is still controversial. While Seeman & Lee (1975) reported that neuroleptic dopamine-blocking agents inhibited the electrically stimulated release of [$^3$H]-dopamine from rat striatal slices, Farnebo & Hamberger (1971b) found that the neuroleptics pimozide and chlorpromazine enhanced the release of [$^3$H]-dopamine under similar experimental conditions.

At present there is no evidence for the presence of presynaptic $\beta$-adrenoceptors in nerve endings of the central nervous system. Yet, the concentration-dependent increase in [$^3$H]-dopamine release during electrical stimulation of rat striatal slices obtained in the presence of dibutyryl cyclic AMP is compatible with the possibility that presynaptic $\beta$-adrenoceptors are involved in this phenomenon.

Szerb & Somogyi (1973) reported a negative feed-back mechanism for acetylcholine released by electrical stimulation from cerebral cortical slices which appears to be mediated by muscarinic presynaptic receptors.

It is possible that presynaptic regulatory mechanisms for transmitter release may be present for other neurotransmitters in the central nervous system, in addition to those described for noradrenaline, dopamine and acetylcholine. Further knowledge in this area may prove to be of great interest. Characterization of the types of presynaptic receptors involved in these regulatory mechanisms for transmitter release may prove to be very useful in designing new drugs with selective affinities for either the pre- or the postsynaptic receptors.

## Conclusions

In addition to the classical receptors that mediate the responses of the effector organ, specific receptors are present in the outer surface of noradrenergic nerve endings. These presynaptic receptors are involved in the regulation of the release of the transmitter during nerve stimulation.

The presynaptic $\alpha$-adrenoceptors mediate a negative feed-back mechanism which leads to inhibition of transmitter release probably by restricting the calcium available for the excitation-secretion coupling.

The presynaptic $\beta$-adrenoceptors mediate a positive feed-back mechanism which is activated at low frequencies of nerve stimulation leading to an increase in transmitter release. This mechanism appears to be mediated through an increase in the levels of cyclic AMP in noradrenergic nerve endings.

Comparison of the pre- and postsynaptic potencies of $\alpha$-receptor agonists and antagonists indicates that the pre- and the postsynaptic $\alpha$-adrenoceptors are not identical.

In addition to the presynaptic $\alpha$- and $\beta$-adrenoceptors, other presynaptic receptors have been described: inhibitory dopamine, opiate and muscarinic receptors. Also the inhibition of release induced by adenosine and by prostaglandins of the E series may be linked to specific presynaptic receptors.

Some of these presynaptic receptors are also present in nerve endings in the central nervous system, like the $\alpha$-adrenoceptor and opiate inhibitory receptors.

Agonists and antagonists with high affinity for presynaptic receptors may elicit pharmacological effects which are due to changes in transmitter release. Such drugs may open new possibilities in therapeutics.

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# EFFECTS OF ASPIRIN-LIKE DRUGS ON CANINE GASTRIC MUCOSAL BLOOD FLOW AND ACID SECRETION

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1 The effects of aspirin, paracetamol and benorylate were studied on gastric mucosal blood flow (MBF) and acid secretion in canine denervated gastric pouches.

2 Aspirin 20 mM in the unstimulated pouch had no effect; pentagastrin-stimulated acid output, but not MBF, was reduced. Aspirin buffered to pH 6 was ineffective.

3 Aspirin 3–50 mg/kg reaching the pentagastrin-stimulated pouch through the blood, increased acid secretion and MBF, but the MBF : secretion ratio was variably affected.

4 Paracetamol (10 or 20 mg/kg i.v., or 20 mM in the pouch) or benorylate (280 mg/kg orally) mainly had little effect.

5 Circular muscle strips from dog arteries were contracted by prostaglandins $E_2$, $F_{1\alpha}$ or $F_{2\alpha}$, and often slightly by indomethacin, but prostaglandin $E_1$ produced variable effects.

6 These results do not favour the view that aspirin causes gastric bleeding in dogs by breakdown of blood vessels due to ischaemia following mucosal vasoconstriction.

## Introduction

Aspirin and other non-steroidal anti-inflammatory drugs cause gastric mucosal erosions and occasionally severe haemorrhage. They also inhibit prostaglandin synthetase, as first shown by Ferreira, Moncada & Vane (1971), Smith & Willis (1971), and Vane (1971). Virchow (1856) proposed a vascular aetiology of peptic ulceration, and it has been suggested that the vascular changes might be due to inhibition of prostaglandin synthesis (Bennett, Stamford & Unger, 1973; Main & Whittle, 1973). Thus, if prostaglandins maintain vasodilatation, removal of this effect by aspirin might cause ischaemia; breakdown of blood vessels might then occur, particularly in areas already damaged by other means. This suggestion was strengthened by the report that aspirin bathing the gastric mucosa of vagally denervated fundic pouches in unstimulated dogs substantially reduced mucosal blood flow (MBF) (O'Brien & Silen, 1973). However, Augur (1970) had previously found little effect, whereas Lin & Warrick (1974), using pentagastrin-stimulated dogs, found increased MBF with aspirin. The importance of this problem led us to re-investigate it, and to study the effect of prostaglandins and indomethacin (a more potent aspirin-like drug) on isolated mesenteric arteries of the dog.

## Methods

Twelve mongrel bitches (12–23 kg) with well-established Heidenhain pouches were used not more than once weekly; they were not used when on heat. Food was withheld for 18 h before experiments, but water was allowed. Mucosal blood flow (MBF) (ml/min) was estimated by clearance of radioactive aniline (Curwain & Holton, 1973), and pouch acid secretion (μmol $H^+$/min) was measured by titration against 0.1 M NaOH with phenolphthalein as indicator. Where appropriate, acid output, MBF and changes in their ratio were calculated. In some experiments gastric acid secretion was stimulated to between 30 and 70% of maximum, by the infusion of pentagastrin ($1–4\ \mu g\ kg^{-1}\ h^{-1}$) (Peptavlon, ICI), mixed with the aniline solution, into a leg vein at 1 ml/min throughout the experiment. The infusion (with or without pentagastrin) was begun 45–60 min before observations were made, to allow stabilization of secretion and equilibration of aniline between the various body compartments. Gastric juice was then collected in 15-min periods, and normally 6 collections were made before administration of the test drug into the main stomach, intravenously or into the pouch. Three consecutive 15-min periods before drug administration were averaged (control value). The 15-min period after administration of a single bolus of drug into the main stomach or intravenously was ignored, to give time for drug effects to occur, and the average of the next three readings (test value) was expressed as percentage of control.

With drugs added to the pouch for 30 min in dogs stimulated with pentagastrin, the test values were the mean of three 15-min periods following the 30-min administration period. The pouch was connected to a reservoir and filled with 15–25 ml of the solution described later. Drug concentration in the pouch was kept fairly constant by replacing 5 ml of the contents with fresh solution every 10 min via a small tube which was also used to allow escape of air when the pouch was being filled. The intraluminal pressure was kept at about 2 cm water. In resting pouches, clearance measurements were made during the instillation period from the combined contents (removed each 30 min) and the washings.

### *Drug solutions*

Aspirin or paracetamol 20 mM were dissolved in distilled water 5 min before administration. The pH of the 20 mM aspirin solution was 2.9–3.1; 20 mM acetic acid was used as a control. Aspirin 20 mM in Sorensen's phosphate buffer 0.67 M, pH 6.0 formed buffered aspirin. When aspirin was studied without secretory stimulation 80 mM HCl was instilled into the pouch throughout to prevent back-diffusion of aniline (Curwain & Holton, 1973) and aspirin (20 mM) was instilled for 30 min as a solution usually in 80 mM HCl.

Small volumes were desirable for oral administration to the main stomach, and aspirin was therefore made up in a form similar to mixture of soluble aspirin BPC (aspirin : sodium carbonate : citric acid 13 : 5 : 3 by weight mixed with water in a pestle and mortar). Doses up to 50 mg/kg could therefore be given in less than 20 ml syringed into the dog's mouth while holding the head up. Control experiments showed that the vehicle had no effect on MBF, acid output or the subsequent titration of acid. Benorylate 40% w/v was administered as Benoral suspension (Sterling Winthrop).

Aspirin for intravenous administration was dissolved aseptically in sterile distilled water. Paracetamol was dissolved in 30 ml of the aniline/pentagastrin infusion and infused over 30 minutes. Benorylate is almost insoluble, and unsuitable for intravenous administration.

### *Studies on dog isolated arteries*

Arteries were obtained from anaesthetized greyhounds and strips of circular muscle were prepared by cutting spirally. The preparations (gastric artery, 12 strips from 7 specimens, root mesenteric or splenic arteries, 1 specimen each) were set up under a load of 0.5 g in Krebs solution at 37°C bubbled with 5% $CO_2$ in $O_2$, and isotonic responses to prostaglandins $E_1$, $E_2$, $F_{1\alpha}$ and $F_{2\alpha}$ determined before and after indomethacin 1–2 µg/ml or (root mesenteric artery only) aspirin 70 µg/ml. The composition of the Krebs solution was as follows (g/l): NaCl 7.1, $CaCl_2.6H_2O$ 0.55, KCl 0.35, $KH_2PO_4$ 0.16, $MgSO_4.7H_2O$ 0.29, $NaHCO_3$ 2.1 and dextrose 1.0.

Results are expressed as means ± s.e., and are analysed statistically by the *t* test for paired data where appropriate, unless stated otherwise. All probability values refer to 2-tailed tests.

## Results

### *Effect of aspirin in the pouch on mucosal blood flow and acid output in unstimulated dogs*

The acid recovered from the pouch after instillation of 80 mM HCl for 30 min was 2.7 ± 1.3% more than the blank control titres, indicating a low basal secretion (8 measurements in 4 dogs). Aspirin (20 mM in 80 mM HCl, instilled into the Heidenhain pouch for 30 min) did not significantly change MBF (4.5% ± 10.5), but only 94.3% of the instilled acid was recovered, indicating some back-diffusion of acid (4 experiments in 4 dogs followed for 90 min from the beginning of the instillation). In 2 of these dogs aspirin was also given in distilled water preceded and followed by 80 mM HCl (1.4 and 13% increase in MBF). In 3 further experiments with 3 dogs, aspirin (10 mg/kg i.v.) given during instillation of 80 mM HCl had a variable effect on MBF (–34 to 105% change); the large variations were probably because of the low and variable blood flow under basal conditions.

### *The effect of aspirin, paracetamol or benorylate on pentagastrin-stimulated acid secretion and mucosal blood flow*

Aspirin instilled into the secreting pouch (20 mM for 30 min) reduced the acid secretory output by 22 ± 6% and the concentration by 7.4 ± 2.8% (both $P < 0.05$; 7 experiments in 5 dogs). However, MBF did not change and its ratio to acid output rose by 19 ± 8%. Aspirin buffered to pH 6.0 before instillation did not significantly affect secretory output (–13 ± 20%) or acid concentration (11 ± 7%, $P > 0.1$) (4 experiments in 4 dogs). No consistent changes in MBF, acid output or concentration occurred in control experiments with 20 mM acetic acid or Sorensen's pH 6 buffer (both 3

**Table 1** Oral (into main stomach) or intravenous aspirin increased pentagastrin-stimulated acid output in conscious Heidenhain-pouch dogs but variably affected the ratio mucosal blood flow (MBF) : acid output (MBF/Acid)

| *Dog No.* | *Dose (mg/kg orally)* | *% Increase in acid output* | *% Increase in MBF* | *% Change in MBF/Acid* | *Dog No.* | *Dose (mg/kg i.v.)* | *% Increase in acid output* | *% Increase in MBF* | *% Change in MBF/Acid* |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 20 | 18 | 18 | 0 | 5 | 3 | 22 | 17 | −4.1 |
| 2 | 20 | 116 | 123 | +3.2 | 3 | 10 | 19 | 15 | −3.4 |
| 3 | 20 | 95 | 80 | −7.7 | 2 | 10 | 3.3 | 3.5 | +0.1 |
| 1 | 33 | 20 | 21 | +0.8 | 2 | 15 | 70 | 94 | +14.1 |
| 4 | 50 | 104 | 95 | −4.5 | 3 | 20 | 68 | 90 | +13.0 |
| 2 | 50 | 72 | 73 | +0.5 | 4 | 20 | 25 | 27 | +1.6 |
| 3 | 50 | 106 | 59 | −22.8 | 2 | 20 | 52 | 62 | +6.5 |
|  |  |  |  |  | 6 | 20 | 34 | 35 | +0.7 |
|  |  |  |  |  | 4 | 44 | 55 | 56 | +0.6 |
|  |  |  |  |  | 2 | 50 | 113 | 103 | −4.7 |

Six dogs were used: 4 received oral aspirin and 5 received intravenous aspirin.

experiments in 3 dogs). In all 17 experiments in 6 dogs, aspirin 3–50 mg/kg orally (i.e. into the main stomach) or intravenously increased acid output (3.3–116%) and MBF (3.5–123%) over a period of 120–135 min following the dose. The ratio of MBF to acid output was variably affected (Table 1). Paracetamol (20 mM) in the pouch had no significant effect on acid output (9.4 ± 5% $0.1 > P > 0.05$), acid concentration (4.8 ± 3.3% $P > 0.1$) or MBF (15 ± 10% $P > 0.1$) (4 experiments in 4 dogs). Paracetamol (10 or 20 mg/kg i.v.; 3 experiments in 3 dogs) had no marked effect on acid output (range −1.7 to −11%) but MBF rose 7.5–47%. With benorylate 280 mg/kg orally acid output increased (by 57 ± 34%) in each of 4 experiments 15–45 min after administration ($0.1 > P > 0.05$) and then declined to previous levels, but the MBF : acid secretion ratio did not alter significantly (0.6 ± 9%).

No bleeding from the pouch was visible with instilled drugs, but on two occasions dogs vomited some bloody gastric juice 1.5 h after oral administration of 50 mg/kg aspirin. The effects of aspirin on pouch secretion began within 15 min of administration.

### *Arterial strips*

At the start of the experiment, each strip was made to contract to noradrenaline 150–600 ng/ml. The responses to prostaglandins $E_2$, $F_{1\alpha}$ and $F_{2\alpha}$ were similar before and after giving indomethacin (1–2 μg/ml). Prostaglandins $E_2$ and $F_{2\alpha}$ (50–300 ng/ml) always caused contraction, but lower concentrations were ineffective; prostaglandin $F_{1\alpha}$ (0.3–3 μg/ml) had no effect or caused contraction. Prostaglandin $E_1$ (0.03–1.5 μg/ml) had a variable effect, causing small relaxations, small contractions or no effect when given before indomethacin. Indomethacin 1–2 μg/ml often increased tone, but the effect was very slight. Prostaglandin $E_1$ given in the presence of indomethacin usually caused a relaxation, but this seemed unlikely to be due substantially to the raised tone (Figure 1). The mesenteric artery was initially unaffected by prostaglandin $E_2$ (25–

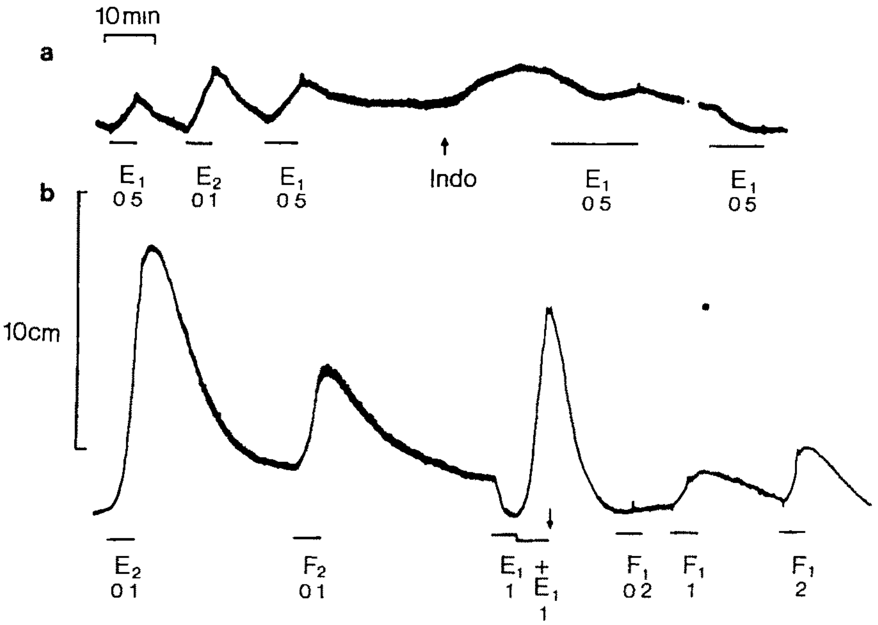

Figure 1 Dog spiral artery strip. (a) Prostaglandin $E_1$ ($E_1$) or prostaglandin $E_2$ ($E_2$) initially evoked contractions. Indomethacin 2 μg/ml (Indo) at the arrow (and re-added with every change of bath fluid) temporarily increased the tone. Subsequent doses of prostaglandin $E_1$ were inhibitory, even when the tone was at a pre-indomethacin level. (b) Follows immediately from (a), with indomethacin still present. Prostaglandins $E_2$ or $F_{2\alpha}$ ($F_2$) caused contraction. Prostaglandin $E_1$ in double the dose used previously was inhibitory, but quadruple the dose was excitatory. Indomethacin was washed out at the arrow. Prostaglandin $F_{1\alpha}$ ($F_1$) caused dose-dependent contractions. Contact times of prostaglandin indicated by solid lines. Numbers below trace indicate μg/ml. The minute lines on the trace were due to air bubbles.

50 ng/ml). After aspirin 70 µg/ml, which slightly increased tone, prostaglandin $E_2$ or $F_{2\alpha}$ (50–100 ng/ml caused contraction but prostaglandin $E_1$ (100 ng/ml) caused relaxation.

## Discussion

We were unable to produce substantial changes in MBF with aspirin instilled into the non-secreting Heidenhain pouch. This is in contrast to the marked reduction obtained by O'Brien & Silen (1973), but similar to the findings of Augur (1970). During stimulation of secretion with pentagastrin, administration of 20 mM aspirin into the pouch had no effect on mucosal blood flow, whereas Lin & Warrick (1974), using 28 mM aspirin in 0.1 M HCl, found an increase of 25–30%. They and others (e.g. Chvasta & Cooke, 1972) obtained gastric mucosal bleeding which was absent in our studies. Nevertheless, like Lin & Warrick (1974) we found that aspirin reduced acid output from the secreting pouches. We confirm the findings of numerous investigators that aspirin causes back-diffusion of $H^+$ through the gastric mucosa (e.g. Davenport, 1964; O'Brien & Silen, 1973; Lin & Warrick, 1974; 1975). Buffering the aspirin to pH 6 prevented this effect in our experiments, presumably because absorption of acetylsalicylic acid was inhibited and there was little acid to diffuse back. Aspirin given intravenously or into the main stomach during pentagastrin infusion increased acid output from the pouch. This is consistent with an inhibitory role of prostaglandins in gastric secretion (Robert, Nezamis & Phillips, 1967); removal of the inhibition by aspirin would increase acid secretion, and the rise in output would be only partly offset by loss of acid by back-diffusion following mucosal damage. Aspirin also increased MBF, presumably secondarily to increased secretion or to back-diffusion of $H^+$ (see later), although the ratio of increased MBF to secretion was variable.

The reason why the results of Augur (1970) and our results differ from those of O'Brien & Silen (1973) is not clear. The latter authors drew attention to their antrectomy procedure, and suggested that circulating gastrin might affect the vascular responses in the pouch mucosa. In both Augur's and our experiments the dogs were not antrectomized, so that our experiments were more 'physiological'. Another difference is that only O'Brien & Silen instilled polyethylene glycol into the pouch. Neither Augur nor O'Brien & Silen stated the sex of animals used; ours were female. Another possible explanation is that aspirin might affect processes which have different effects on MBF. Perhaps prostaglandin within the canine gastric mucosa normally inhibits acid secretion (Robert *et al.*, 1967) but the tendency for aspirin to cause vasoconstriction by inhibiting prostaglandin synthesis in blood vessels is offset by stimulation of blood flow secondary to increased acid secretion, or by vasodilatation caused by back-diffusion of $H^+$ (Ritchie, 1975; Whittle, 1976). This argument seems plausible with pentagastrin stimulation since aspirin increased acid output. It seems less likely in the resting pouch where aspirin did not change the zero output, but perhaps secretion occurred which was lost by back-diffusion. Benorylate or paracetamol, in amounts respectively four times higher and the same as used in man, mainly had little effect on the stomach, and it might be relevant that in man these drugs produce little or no gastric bleeding. However, it is not known if they inhibit prostaglandin synthesis in dog stomach; prostaglandin synthetases vary in their sensitivity to drugs.

Formation of prostaglandin-like material by dog mesenteric arteries is inhibited by indomethacin (A. Robert, personal communication). Like aspirin, this drug too causes gastric bleeding and back-diffusion of $H^+$ in the dog (Lin & Warrick, 1975). It also produces vasoconstriction in rat gastric mucosa (Main & Whittle, 1975) and at other sites (human cerebral and conjunctival vessels, Sicuteri, Michelacci & Anselmi, 1965; Vecchio & Fontana, 1965). Similarly, we obtained a slight contraction with indomethacin in canine arterial strips, and it seems likely that a similar effect occurs in the dog gastric microvasculature. However, *in vivo* changes in other factors such as acid secretion might offset the vasoconstriction. Of the prostaglandins tested, only prostaglandin $E_1$ caused relaxation, in agreement with the findings (Shehadeh, Price & Jacobson, 1969) that infusion of prostaglandin $E_1$ 0.05–1 µg $kg^{-1}$ $h^{-1}$ intra-arterially increased mesenteric artery blood flow in anaesthetized dogs, whereas prostaglandin $F_{2\alpha}$ 0.1–1 µg $kg^{-1}$ $h^{-1}$ usually reduced flow. Thus, if the weak vasoconstrictor effect of indomethacin which occurred in some strips was due to inhibition of locally produced prostaglandins, perhaps the formation and effect of prostaglandin $E_1$ predominated in those arteries. Alternatively, other prostaglandins or thromboxanes might be produced, or indomethacin might act on another pathway such as inhibition of cyclic adenosine 3',5'-monophosphate phosphodiesterase (Flower, 1974).

It is possible that in rats, aspirin causes gastric bleeding by inhibiting prostaglandin synthetase and causing vasoconstriction (Main & Whittle, 1973). Our results suggest that this is not so in the dog, although a tendency to cause vasoconstriction might be offset by secretory stimulation and by back-diffusion of $H^+$. It might be relevant that we did not observe bleeding from the unstimulated pouch with locally administered aspirin, but the dose was sufficient to produce secretory changes when given by other routes during pentagastrin stimulation. What happens in man is not known, but since indomethacin (administered rectally) did not increase human gastric acid secretion during submaximal stimulation with pentagastrin (Bennett *et al.*, 1973) there seems unlikely to be a secretory stimulus to offset any direct indomethacin-induced vasoconstriction that might occur. The answer must await clinical studies on gastric mucosal blood flow.

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# FLUVOXAMINE, A SPECIFIC 5-HYDROXYTRYPTAMINE UPTAKE INHIBITOR

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1 On the basis of both *in vitro* and *in vivo* experiments fluvoxamine has been characterized as a potential anti-depressant drug with almost exclusively 5-hydroxytryptamine (5-HT) uptake inhibiting properties.

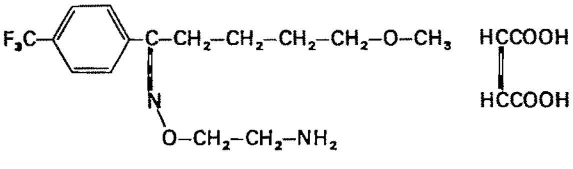
2 Fluvoxamine is effective in inhibiting 5-HT uptake by blood platelets and brain synaptosomes. Due to inhibition of the membrane pump the compound prevents 5-HT depletion by the tyramine-derivatives H 75/12 and H 77/77. As a result of the interference with the neuronal re-uptake mechanism for 5-HT, fluvoxamine produces a decreased 5-HT turnover in the brain. Effects of 5-hydroxytryptophan (5-HTP) are potentiated in mice and in combination with pargyline, fluvoxamine induces 5-HT-like behavioural effects.

3 In contrast to tricyclic antidepressants, noradrenaline uptake processes are either unaffected or only slightly inhibited by fluvoxamine. The noradrenaline depleting effects of tyramine derivatives are not influenced by fluvoxamine. Reserpine effects, such as ptosis are affected only at very high doses of the test compound. The antagonism by fluvoxamine of the reserpine-induced lowering of the pentamethylenetetrazole convulsive threshold can be regarded as due to an effect upon 5-HT uptake. In contrast to the effects of desmethylimipramine and imipramine, no stimulatory effects are found in rats when rapidly acting reserpine-like compounds are given following a dose of fluvoxamine.

## Introduction

Growing support has been accumulating during recent years for the view that a disturbance in 5-hydroxytryptamine (5-HT) metabolism may be an important contributory factor in the development of depressive illness. It is to be expected that in the next few years significant improvement of the diagnostic classification of depressive disorders by biochemical means will clarify which subtypes of depression will benefit most by a normalization of 5-HT metabolism. On this basis, it can be postulated that, in addition to the available tricyclic antidepressant compounds which primarily affect the noradrenaline re-uptake, there is a need for a drug with a specific inhibitory effect on neuronal 5-HT re-uptake.

Compounds in the series of 2-aminoethyloximethers of aralkylketones possess inhibiting properties both with regard to neuronal noradrenaline (NA) and 5-HT re-uptake. The relative activity with respect to NA and 5-HT re-uptake processes is quite structure-specific. Fluvoxamine was selected from the series as being a compound with a rather high 5-HT re-uptake inhibiting activity but which has little effect on NA re-uptake.



Fluvoxamine

(E)-5-methoxy-4′-(trifluoromethyl)valerophenone

O-(2-aminoethyl)oxime maleate (1 : 1).

In this paper pharmacological and biochemical studies are presented which focus primarily on the characterization of the compound, with respect to inhibition of the neuronal re-uptake processes of 5-HT and NA.

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## Methods

### *Potentiation of 5-hydroxytryptamine: inhibition of uptake processes*

*Inhibition of 5-hydroxytryptamine uptake by blood platelets.* A blood platelet suspension in Sneddon's incubation medium (Sneddon, 1969) was prepared from guinea-pig blood. To ultramicrotubes containing 100 µl of a platelet-suspension, 10 µl of various concentrations of solutions of the test compound were added. The tubes were allowed to stand for 5 min at 37°C. After the addition of 20 µl of [$^{14}$C]-5-HT solution giving a final concentration of $5 \times 10^{-6}$ M, the mixtures were incubated for 60 min at 37°C with occasional mixing. 5-HT uptake was terminated by cooling the tubes and immediate separation of the platelets by rapid centrifugation (Beckman-Sanz, micro-angle, 10,000 *g* for 4 minutes). The supernatant was decanted and the pellet washed twice with ice-cold medium and centrifuged. The resulting pellet was resuspended in 200 µl of a 4% solution of Triton-X-100 for complete lysis. A 150 µl sample was taken for liquid scintillation counting. Using as references the zero-time and the incubated control values, the $pI_{50}$-values, indicating the negative logarithm of the molar concentration causing 50% inhibition of the uptake, were calculated graphically for the test compounds.

*Inhibition of 5-hydroxytryptamine uptake by rat brain synaptosomes.* Male adult Wistar rats (240–300 g) were killed by cervical dislocation, their brains rapidly removed and a crude synaptosomal fraction ($P_2$) prepared by homogenization and differential centrifugation by the method of Whittaker (1969); 0.2 ml portions of the resuspended $P_2$-pellet, equivalent to approximately 25 mg original brain wet weight were pre-incubated for 4 min at 37°C. Tritium labelled 5-HT was then added and the incubation was continued for another 5 min (final volume 1.0 ml, inhibitor concentration $10^{-6}$ M, 5-HT concentrations $10^{-6}$, $2 \times 10^{-7}$ and $6.7 \times 10^{-8}$ M). The incubation was stopped by cooling followed by filtration (Sartorius filter, pore size 0.6 µm). The residue was washed and the filter containing synaptosomes dissolved with 2 ml of 2-ethoxyethanol. The radioactivity was measured by liquid scintillation counting. Uptake data, corrected for 'uptake' at 0°C, were evaluated by construction of a Lineweaver-Burk plot. As a measure for the relative activity of the uptake inhibition, the $K_i$-value was calculated according to the formula $K_i = (i/K_{mi}/K_m - 1)$ where $i$ is the molar concentration of the inhibitor and $K_m$ and $K_{mi}$ are the intercepts on the abscissa scale of the control and the inhibitor graphs respectively.

For displacement studies the crude synaptosomal fraction ($P_2$) was preloaded with [$^3$H]-5-HT by incubation at a concentration of $5 \times 10^{-8}$ M for 20 min at 37°C. After centrifugation at 14,000 *g* for 10 min at 4°C the pellet was resuspended in fresh incubation medium. Displacement of [$^3$H]-5-HT was brought about by addition of unlabelled amine to aliquot samples and further incubation during 15 min at 37°C (final volume 1.0 ml, synaptosomal protein content approximately 1 mg, 5-HT concentrations $10^{-7}$, $10^{-6}$ and $10^{-5}$ M). The effect of the uptake inhibitor on the displacement was studied by incorporation into the incubation medium of the test compound at a final concentration of $10^{-6}$ or $10^{-5}$ M. The content of [$^3$H]-5-HT remaining in the synaptosomes was determined as described above and was expressed as a percentage of the content in synaptosomes which were incubated without addition of unlabelled amine.

An estimate of the *in vivo* inhibition of the uptake process was obtained by studying the uptake properties *in vitro* of synaptosomes isolated from rats that had been treated with the inhibitor compounds. The test compound was administered intraperitoneally to male Wistar rats (250–280 g) at a dose of 25 mg/kg. Untreated animals were used as controls. The treated animals were killed after 30, 60 and 90 min respectively. The cerebral hemispheres were removed and homogenized in 6 ml of 0.32 M sucrose per g tissue as described above. Heavy debris was removed by centrifugation for 10 min at 1,000 *g*. The supernatant was used as the crude synaptosomal fraction ($SN_1$). Aliquots of the supernatant (2.0–2.6 mg protein) were preincubated for 4 minutes. 5-HT uptake was estimated by incubation with [$^3$H]-5-HT at concentrations of $5 \times 10^{-8}$ and $10^{-7}$ M as described. The results were expressed as pmol 5-HT taken up per mg of synaptosomal protein during 5 minutes.

*Inhibition of 5-hydroxytryptamine release by the tyramine derivatives H 75/12 and H 77/77.* H 77/77 was administered intramuscularly to male Wistar rats (220–270 g) in two doses of 12.5 mg/kg; there was a 2 h interval between doses. The test compound was injected intramuscularly at a dose of 25 mg/kg 30 min before each H 77/77 injection. H 75/12 was injected with the same dosing schedule but using two doses of 25 mg/kg. Four hours after the first injection of the releasing substance the animals were killed, the brain removed and homogenized in acid *n*-butanol. The extract was analysed spectrofluorimetrically for 5-HT according to a modification of the method of Shore & Olin (1958).

*Effect upon 5-hydroxytryptamine turnover in rat brain.* The compound was administered intramuscularly to rats in a dose of 25 mg/kg together with an intraperitoneal injection of 200 mg/kg probenecid; 2 h later a second dose of probenecid was injected. Four hours after the first administration, the animals were killed. The brain was removed, cooled, and the cerebellum separated. The tissue was homogenized in acid *n*-butanol; 5-HT and 5-hydroxyindoleacetic acid

(5-HIAA) were separated by the normal acid extraction procedure. The cerebellum homogenate was used as a tissue blank. The concentration of 5-HT and 5-HIAA was measured fluorimetrically using an Aminco Bowman spectrofluorimeter. Correction factors for the calculation of the tissue concentration were obtained by performing the total procedure simultaneously with standard solutions.

The turnover rate was derived from the formula:

$$\frac{(\text{5-HIAA})\text{ treated} - (\text{5-HIAA})\text{ control}}{4 \times 191} \times 1000\ \text{nmol g}^{-1}\ \text{h}^{-1}$$

In similar experiments the activity of fluvoxamine was demonstrated after oral application. The test compound was administered orally 15 min before the administration of probenecid at a dose of 200 mg/kg intraperitoneally. The animals were killed 2 h later, the total brain was removed and homogenized in acid *n*-butanol. After the usual separation procedures, 5-HIAA was determined fluorimetrically as its orthophthalicaldehyde fluorophor (Neff, Tozer & Brodie, 1967; Curzon & Green, 1970; Maickel, Cox, Saillant & Miller, 1968).

*Potentiation of 5-hydroxytryptophan effects in mice.* The test compound was administered orally in a range of doses to isolated male Swiss albino mice (5 mice per test dose), 1 h or 6 h respectively before the intraperitoneal injection of 150 mg/kg of DL-5-hydroxytryptophan (5-HTP), this being a threshold dose of 5-HTP. Thirty min after the 5-HTP injection, the mice were individually observed and the following parameters scored: stereotyped head-searching movements (on a 0 to 4 scale), hind limb abduction (0 to 2), tremor (0 to 4), escape tendency (0 or 1), fore limb clonus (0 to 2), lordosis (0 to 2). From the results, the $ED_{50}$ values were determined by plotting log dose against total group score on a linear scale. The $ED_{50}$ value is the dose required to potentiate the 5-HTP effect to 50% of the maximal score.

*Potentiation of pargyline effects in mice.* The induction of 5-HT-like effects was studied by administering fluvoxamine, in a series of doses, 4 h after administration of pargyline at a dose of 100 mg/kg intraperitoneally. The various parameters described in the previous section were scored 1 h after administration of the test compound, and the $ED_{50}$ value was again determined graphically.

*Antagonism of the reserpine-induced lowering of the pentamethylenetetrazole convulsive threshold.* The test compound or 1% tragacanth was administered orally to groups of 10 male mice, 1 h before subcutaneous injection of reserpine (5 mg/kg); 2 h after the reserpine injection, a 0.45% solution of pentamethylenetetrazole was injected intravenously at the rate of 0.05 ml every 10 s (Orloff, Williams & Pfeiffer, 1949). The volume of solution producing the tonic extensor component of the convulsion was noted.

*Potentiation of noradrenergic effects—inhibition of uptake processes*

*Inhibition of noradrenaline and dopamine uptake by rat brain synaptosomes.* The effect of fluvoxamine on NA and dopamine uptake by rat brain synaptosomes was studied in the same manner as described for 5-HT.

Inhibition of NA and dopamine was also studied by displacement experiments as described for 5-HT.

The *in vivo* inhibition of NA and dopamine uptake processes was also studied with synaptosomes prepared from animals treated with the antidepressant compound. These experiments were performed in the same way as those described for 5-HT except, that [$^3$H]-NA and [$^3$H]-dopamine were used in place of [$^3$H]-5-HT.

*Inhibition of noradrenaline release by H 75/12 and H 77/77.* The method and dosage regimen used were as described for the experiments with these tyramine derivatives on 5-HT release. The acid *n*-butanol extract of brain was analysed for NA by a modification of the method of Welch & Welch (1969).

*Potentiation of noradrenaline effects on rat vas deferens.* The test was performed according to the method of Ursillo & Jacobson (1965).

*Antagonism of tetrabenazine-induced ptosis in mice.* The test compound was administered intraperitoneally or orally to groups of 5 male mice in a range of doses. After a time interval of 45 min tetrabenazine was injected subcutaneously at a dose of 80 mg/kg. Ptosis was scored 45 min after tetrabenazine administration by use of a ptosis chart (Rubin, Maline, Waugh & Burke, 1957). The dose ($ED_{50}$) of the test compound required to reduce the ptosis score to half that of the control tetrabenazine group was determined graphically by plotting responses on a probit scale against test doses on a logarithmic scale.

*Antagonism of tetrabenazine-induced ptosis and induction of compulsive hyperactivity in rats.* The method was as described in the preceding paragraph except that male albino rats were employed, a dose of 40 mg/kg of tetrabenazine was used and ptosis was scored by 2 observers at 90 min after tetrabenazine administration. During this test, the animals were also scored for the appearance of a hyperactive state. On the basis of the number of rats per dose group showing such behaviour, the hyperactivity $ED_{50}$ was calculated (Horn, 1956).

### *Drugs*

The following drugs were used: pargyline (Abbott Labs); tetrabenazine (Hoffman La Roche); chlorimipramine, desmethylimipramine (DMI), imipramine and reserpine (Ciba Geigy); probenecid (Merck, Sharp & Dohme); 5-HTP and 5-HT (Koch Light Labs); H 75/12 (Labkemi); H 77/77 (Axel Kistner).

5-Hydroxytryptamine creatinine sulphate [side chain 2-$^{14}$C], 56 mCi/mmol was obtained from Radiochemical Centre, Amersham.

The following radiochemicals were obtained from NEN chemicals: (—)-noradrenaline[7-$^3$H], 6.41 Ci/mmol; 3,4-dihydroxyphenylethylamine ethyl-[2-$^3$H], 5.9 Ci/mmol; 5-hydroxytryptamine bioxalate[1,2-$^3$H], 1.0 Ci/mmol.

## Results

### *Potentiation of 5-hydroxytryptamine effects: inhibition of uptake processes*

*Inhibition of 5-hydroxytryptamine uptake by blood platelets.* The activity of fluvoxamine in inhibiting 5-HT uptake by blood platelets was comparable to the activity of imipramine (Table 1). Both compounds were markedly more active than tricyclic secondary amines such as DMI.

*Inhibition of 5-hydroxytryptamine uptake by rat brain synaptosomes.* The Lineweaver-Burk plots for fluvoxamine, imipramine and DMI demonstrated a common intercept on the ordinate indicating no change in $V_{max}$. From this it may be concluded that the three compounds caused a competitive inhibition of the uptake process. Fluvoxamine showed the highest activity (Table 2).

**Table 1** Inhibition of 5-hydroxytryptamine uptake in guinea-pig blood platelets

| *Compound* | $pI_{50}$ |
|---|---|
| Fluvoxamine | 6.4 ± 0.20 (4) |
| Chlorimipramine | 8.2 ± 0.55 (3) |
| Imipramine | 6.8 ± 0.12 (14) |
| Desmethylimipramine | 4.9 ± 0.20 (2) |

Values are the means ± s.e. mean; the number of determinations is given in parentheses. The values of $pI_{50}$, indicating the negative logarithm of the molar concentration causing 50% inhibition of the uptake, were obtained from the graph relating log dose and percentage inhibition.

Increasing concentrations of unlabelled 5-HT produced an increasing release of labelled 5-HT from preloaded rat brain synaptosomes. In the concentrations used, fluvoxamine and imipramine themselves caused a small but significant release. Under these experimental conditions fluvoxamine inhibited the release of radioactive material by 5-HT up to a 5-HT concentration of $10^{-6}$ M. Fluvoxamine was in this respect at least 10 times more active than imipramine (Table 3).

Fluvoxamine or imipramine administration to rats 30, 60 or 90 min before they were killed, produced a significant inhibition of the uptake process of the synaptosomes when studied *in vitro*. The inhibitory effect was already apparent after 30 min and did not diminish during the longer time-intervals. In accordance with the results obtained in the *in vitro* experiments, fluvoxamine showed a markedly stronger inhibition than imipramine (Table 4).

*Inhibition of 5-hydroxytryptamine release by the tyramine derivatives H 75/12 and H 77/77.* Both H 75/12 and H 77/77 produced a marked depletion of brain 5-HT. Fluvoxamine, given twice at 25 mg/kg was very active in inhibiting the depleting action of both compounds. A clear protective effect against depletion by H 75/12 was found for imipramine at the same dose though the inhibitory effect was much less than for fluvoxamine. DMI caused a significant but rather small inhibition of the depletion by H 77/77 (Table 5).

*Effect upon 5-hydroxytryptamine turnover rate.* Probenecid produced a marked increase in the brain 5-HIAA content, compared to the untreated control animals, by inhibition of the transport of 5-HIAA out of the brain. The turnover rate of 5-HT calculated from these results, amounted to 1.31 nmol g$^{-1}$ h$^{-1}$, a value closely corresponding to values reported by other authors (Neff, Lin, Ngai & Costa, 1969).

**Table 2** Inhibition of 5-hydroxytryptamine (5-HT) uptake in rat brain synaptosomes *in vitro*

| *Compound* | $K_i \times 10^6$ *M* |
|---|---|
| Fluvoxamine | 0.084 |
| Imipramine | 0.23 |
| Desmethylimipramine | 1.2 |

Crude synaptosome fraction was incubated for 5 min with [$^3$H]-5-HT at final concentrations of $10^{-6}$, $2 \times 10^{-7}$ and $6.7 \times 10^{-8}$ M; inhibitor concentration $10^{-6}$M. Uptake data plotted as Lineweaver-Burk graph; $K_i$ value is calculated according to $Ki = (I/K_{mi}/K_m - 1)$ where $I$ is the molar concentration of the inhibitor and $K_m$ and $K_{mi}$ are the intercepts on the abscissae of the control and inhibitor graphs respectively

**Table 3** Inhibition of displacement of [$^3$H]-5-hydroxytryptamine from rat brain synaptosomes by unlabelled amine.

| *5-HT concentration in incubation fluid (M)* | *[$^3$H]-5-HT content remaining in synaptosomes (% control)* Control | Fluvoxamine $10^{-6}$ M | Fluvoxamine $10^{-5}$ M | Imipramine $10^{-6}$ M | Imipramine $10^{-5}$ M |
|---|---|---|---|---|---|
| 0 | 100.0 | 91.0 | 82.7 | 90.8 | 86.7 |
| $10^{-7}$ | 77.0 | 84.8 | 83.9 | 82.4 | 87.4 |
| $10^{-6}$ | 24.7 | 80.1 | 84.1 | 42.8 | 74.5 |
| $10^{-5}$ | 18.4 | 63.4 | 78.7 | 22.4 | 34.7 |

Values are the means of two incubation experiments performed in parallel. Crude synaptosomal fraction was preloaded with [$^3$H]-5-HT by *in vitro* incubation. Effect of inhibitor on displacement by unlabelled amine was measured during 15 min incubation.

**Table 4** Inhibition of 5-hydroxytryptamine (5-HT) uptake in rat brain synaptosomes after *in vivo* dosing

| *Compound* | *Uptake of [$^3$H]-5-HT (pmol mg$^{-1}$ synapt. protein 5 min$^{-1}$)* 30 min | 60 min | 90 min |
|---|---|---|---|
| Control | | 6.10<br>±0.14 (12) | |
| Fluvoxamine | 1.25*<br>±0.08 (4) | 1.07*<br>±0.07 (4) | 0.98*<br>±0.10 (4) |
| Imipramine | 3.13*<br>±0.19 (4) | 3.26*<br>±0.60 (4) | 3.02*<br>±0.21 (4) |

Values are the means ± s.e. mean, the number of determinations is given in parentheses. Test compounds were administered intraperitoneally to male rats in a dose of 25 mg/kg. Brains were removed at indicated post-dose times. The crude synaptosomal fraction was incubated for 5 min with [$^3$H]-5-HT in a final concentration of $5 \times 10^{-8}$ M.

* *P* value <0.001 when compared to control group (Student's *t* test).

Fluvoxamine decreased the turnover rate to 0.84 nmol g$^{-1}$ h$^{-1}$. Imipramine also produced a decrease in turnover rate, although to a lesser extent than fluvoxamine. DMI was inactive in this respect (Table 6).

After oral administration, fluvoxamine also greatly reduced 5-HT turnover as was apparent from the very strong inhibition of the increased 5-HIAA accumulation by probenecid, being 62% at a dose of 50 mg/kg and 95% at a dose of 70 mg/kg ($n = 5$).

*Potentiation of 5-hydroxytryptophan behavioural effects.* Fluvoxamine was effective in potentiating the central effects of 5-HT as observed when administered together with 5-HTP. The compound was clearly more active than the most active of the clinically used tricyclic antidepressants, namely chlorimipramine. Imipramine was clearly less active than chlorimipramine while DMI showed no activity.

From the ratio of the ED$_{50}$ values measured 6 and 1 h respectively after administration of the test

**Table 5** Inhibition of 5-hydroxytryptamine (5-HT) release from rat brain by H 75/12 and H 77/77

| *Test compound (2 × 25 mg/kg i.m.)* | *Releasing agent* | *Brain 5-HT content (µg/g tissue)* | P *value* |
|---|---|---|---|
| Control | — | 0.67 ± 0.03 (8) | |
| Fluvoxamine | — | 0.66 ± 0.03 (7) | |
| Imipramine | — | 0.55 ± 0.01 (6) | <0.01 |
| Desmethylimipramine | — | 0.54 ± 0.01 (6) | <0.01 |
| — | H 75/12 | 0.23 ± 0.02 (8) | |
| Fluvoxamine | H 75/12 | 0.53 ± 0.03 (8) | <0.001 |
| Imipramine | H 75/12 | 0.37 ± 0.02 (8) | <0.001 |
| — | H 77/77 | 0.27 ± 0.02 (8) | |
| Fluvoxamine | H 77/77 | 0.51 ± 0.01 (8) | <0.001 |
| Desmethylimipramine | H 77/77 | 0.32 ± 0.02 (8) | <0.05 |

Values are the means ± s.e. mean, the number of determinations is given in parentheses. H 77/77 was injected at two doses of 12.5 mg/kg with a 2 h interval between doses. Test compounds were injected intramuscularly 30 min before each H 77/77 injection. H 75/12 was injected using the same dosing schedule but using two doses of 25 mg/kg.

*P* values refer to comparison with appropriate control group (Student's *t* test).

**Table 6** Effect upon 5-hydroxytryptamine (5-HT) turnover in rat brain

| *Probenecid pretreatment (2 × 200 mg/kg i.p.)* | *Test compound (25 mg/kg i.m.)* | *Brain 5-HIAA conc (µg/g tissue)* | *Turnover rate (nmol $g^{-1}$ $h^{-1}$)* |
|---|---|---|---|
| — | Control | 0.31 ± 0.018 (14) | |
| Probenecid | — | 1.32 ± 0.058 (16) | 1.31 |
| Probenecid | Fluvoxamine | 0.95* ± 0.042 (8) | 0.84 |
| Probenecid | Imipramine | 1.04* ± 0 069 (8) | 0 94 |
| Probenecid | Desmethylimipramine | 1.14 ± 0 043 (8) | 1 08 |

Values for 5-hydroxyindoleacetic acid (5-HIAA) content are the means ± s.e. mean; the number of determinations is given in parentheses. Turnover rate calculated as

$$\frac{\text{(5-HIAA) treated} - \text{(5-HIAA) control}}{4 \times 191} \times 1000 \text{ nmol g}^{-1}\text{ h}^{-1}$$

Test compound administered intramuscularly at same time as first dose of probenecid, second dose of probenecid after 2 h; animals killed 4 h after administration of test compound.

* $P$ value < 0.01 when compared to probenecid control group (Student's $t$ test).

compound, it is apparent that fluvoxamine had a long duration of action (Table 7).

*Potentiation of pargyline effects.* Fluvoxamine induced marked 5-HT-like effects when combined with pargyline. The tricyclic antidepressants showed no activity in this test (Table 8).

*Antagonism of reserpine-induced lowering of the pentamethylenetetrazole convulsive threshold.* Fluvoxamine at oral doses of 10, 20 and 40 mg/kg caused a statistically significant antagonism of the reserpine-induced lowering of the pentamethylenetetrazole convulsive threshold. Chlorimipramine was active in this respect at a dose of 20 mg/kg; imipramine and DMI, however, were inactive at this dose (Table 9).

**Table 7** Potentiation of 5-hydroxytryptophan (5-HTP) effects in mice

| *Test compound* | *Oral $ED_{50}$ (mg/kg)* | |
|---|---|---|
| | *90 min* | *390 min* |
| Fluvoxamine | 36 ± 4.3 (7) | 141 ± 21 (3) |
| Chlorimipramine | 84 ± 6.6 (5) | ~240 (2) |
| Imipramine | 135 ± 18 (4) | |
| Desmethylimipramine | > 320 (3) | |

Values are the means ± s.e. mean; the number of determinations is given in parentheses. Test compound was administered to groups of 5 mice in a range of doses 60 min and 360 min respectively before the administration of an intraperitoneal dose of 150 mg/kg of 5-HTP. Behavioural parameters were scored after a further 30 min: stereotyped headsearching movements on a 0 to 4 scale, hindlimb abduction (0 to 2), tremor (0 to 4), escape tendency (0 or 1), forelimb clonus (0 to 2), lordosis (0 to 2). $ED_{50}$ value, the dose of the test compound required to potentiate the 5-HTP effects to 50% of the maximum score, was obtained from the graph relating log dose and total group score.

*Potentiation of noradrenergic effects: inhibition of uptake processes*

*Inhibition of noradrenaline and dopamine uptake by rat brain synaptosomes.* Fluvoxamine, as well as the tricyclic antidepressants, inhibited the uptake processes of NA and dopamine in synaptosomes from total brains. The Lineweaver-Burk plots indicated a competitive mechanism for the inhibition. Fluvoxamine was almost as active as DMI in this experiment in inhibiting NA uptake, though the effect of both compounds was rather weak.

Increasing concentrations of unlabelled amine

**Table 8** Potentiation of pargyline effects in mice

| *Test compound* | *Oral $ED_{50}$ (mg/kg)* |
|---|---|
| Fluvoxamine | 20 ± 1 2 (4) |
| Chlorimipramine | > 320 (2) |
| Imipramine | > 320 (2) |
| Desmethylimipramine | > 320 (2) |

The values are the mean ± s.e. mean; the number of determinations is given in parentheses. Pargyline was administered intraperitoneally 4 h before orally dosing the test compound to groups of 5 mice. For details of behavioural parameters see footnote to Table 7. $ED_{50}$ value, the dose of the test compound required to induce a behavioural score equal to 50% of the maximum score, was obtained from the graph relating log dose and total group score.

**Table 9** Antagonism of the reserpine-induced lowering of the pentamethylenetetrazole convulsive threshold

| *Test compound* | *Oral dose (mg/kg)* | *Reserpine (5 mg/kg s.c.)* | *Convulsive dose pentamethylenetetrazole (ml 0.45% solution)* |
|---|---|---|---|
| *Experiment 1* | | | |
| Vehicle | — | — | 0.66±0.03 |
| Vehicle | — | Reserpine | 0.18±0.03 |
| Fluvoxamine | 5 | Reserpine | 0.20±0.03 |
| Fluvoxamine | 10 | Reserpine | 0.28±0.05* |
| Fluvoxamine | 20 | Reserpine | 0.39±0.06* |
| Fluvoxamine | 40 | Reserpine | 0.36±0.06* |
| *Experiment 2* | | | |
| Vehicle | — | — | 0.53±0.06 |
| Vehicle | — | Reserpine | 0.17±0.01 |
| Chlorimipramine | 5 | Reserpine | 0.22±0.03 |
| Chlorimipramine | 10 | Reserpine | 0.18±0.01 |
| Chlorimipramine | 20 | Reserpine | 0.28±0.05* |
| Chlorimipramine | 40 | Reserpine | 0.30±0.06* |
| *Experiment 3* | | | |
| Vehicle | — | — | 0.46±0.06 |
| Vehicle | — | Reserpine | 0.18±0.01 |
| Imipramine | 20 | Reserpine | 0.20±0.04 |
| Desmethylimipramine | 20 | Reserpine | 0.19±0.03 |

The values are the mean doses (ml±s.e. mean) of a 0.45% pentamethylenetetrazole solution required to produce tonic extensor seizures. Test compounds were administered orally to groups of 10 mice 1 h before a subcutaneous dose of 5 mg/kg of reserpine. Seizure susceptibility was tested 2 h after reserpine administration by intravenous injection of the pentamethylenetetrazole solution at the rate of 0.05 ml every 10 seconds

* *P* value ⩽0.05 when compared to reserpine-treated control group (Wilcoxon).

produced an increasing release of tritium-labelled NA and dopamine respectively from preloaded rat brain synaptosomes. In the concentrations used, fluvoxamine and DMI caused a small but significant release. Fluvoxamine at a concentration of $10^{-5}$ M inhibited NA displacement with an amine concentration of $10^{-7}$ M; at higher amine concentrations no inhibition was apparent. In contrast DMI at a concentration of $10^{-5}$ M caused a marked inhibition at all three NA concentrations. Fluvoxamine is at least 10 times less active than DMI in these experiments. Neither fluvoxamine nor DMI showed an effect against displacement of dopamine (Table 10).

Both fluvoxamine and imipramine, administered to

**Table 10** Inhibition of displacement of [$^3$H]-noradrenaline and [$^3$H]-dopamine from rat synaptosomes by unlabelled amine

| *Amine concentration in incubation fluid* | *[$^3$H]-noradrenaline content remaining in synaptosomes (% control)* | | | | *[$^3$H]-dopamine content remaining in synaptosomes (% control)* | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Fluvoxamine* | *DMI* | | | *Fluvoxamine* | *DMI* | |
| (M) | Control | $10^{-5}$ M | $10^{-6}$ M | $10^{-5}$ M | Control | $10^{-5}$ M | $10^{-6}$ M | $10^{-5}$ M |
| 0 | 100.0 | 93.6 | 93.2 | 92.8 | 100.0 | 94.9 | 97.0 | 94.4 |
| $10^{-7}$ | 79.3 | 86.7 | 91.6 | 91.2 | 62.6 | 53.6 | 57.7 | 58.9 |
| $10^{-6}$ | 61.4 | 63.1 | 68.1 | 73.5 | 21.6 | 20.1 | 23.9 | 23.8 |
| $10^{-5}$ | 45.4 | 43.8 | 50.4 | 55.8 | 11.5 | 11.0 | 13.8 | 15.1 |

Values are the means of two incubation experiments performed in parallel. Crude synaptosome fraction was preloaded with [$^3$H]-noradrenaline or [$^3$H]-dopamine respectively by *in vitro* incubation. Effect of inhibitor on displacement by unlabelled amine was measured during 15 min incubation.

**Table 11** Effect on uptake of noradrenaline and dopamine by rat brain synaptosomes after *in vivo* dosing

| *Compound* | *Uptake of [$^3$H]-noradrenaline* (pmol mg$^{-1}$ synapt. protein 5 min$^{-1}$) 30 min | 60 min | 90 min | *Uptake of [$^3$H]-dopamine* (pmol mg$^{-1}$ synapt. protein 5 min$^{-1}$) 30 min | 60 min | 90 min |
|---|---|---|---|---|---|---|
| Control | | 3.70 ±0.16 | | | 16.3±0.47 | |
| Fluvoxamine | 3.55 ±0.06 | 3.56 ±0.12 | 3.38* ±0.26 | 16.3±0.84 | 17.1±0.38 | 13.9* ±0.61 |
| Imipramine | 3.36* ±0.13 | 3.26* ±0.18 | 2.91* ±0.37 | 19.2±0.72 | 17.6±0.60 | 15.0 ±1.08 |

Values are the means ± s.e. mean; the number of determinations was 12 for the control group and 4 in all other cases. Test compounds were administered intraperitoneally to male rats at a dose of 25 mg/kg. Brains were removed at indicated post-dose times The crude synaptosome fraction was incubated for 5 min with [$^3$H]-noradrenaline and [$^3$H]-dopamine in a final concentration of $5 \times 10^{-8}$ M.

* *P* value <0.05 when compared to control group (Student's *t* test)

rats at a dose of 25 mg/kg intraperitoneally, caused a significant inhibition of the uptake of noradrenaline by isolated synaptosomes; however in contrast to imipramine, which was active at all three time intervals studied, fluvoxamine exhibited only a small and non-significant effect at 90 min post-dose (Table 11).

A small significant inhibition of 15% of dopamine uptake was found with synaptosomes of animals treated with fluvoxamine 90 min before they were killed.

*Inhibition of noradrenaline release by H 75/12 and H 77/77.* The results of the depleting action of the tyramine derivatives on brain noradrenaline and of the interfering effects of the test compounds are summarized in Table 12. As can be seen from these results H 75/12 and H 77/77 both caused a substantial decrease in brain NA content. Fluvoxamine, in two doses of 25 mg/kg, did not protect against the depleting action of these agents. In contrast, DMI was very effective in preventing the depeletion of NA by H 77/77. None of the test compounds administered alone affected the NA content of the brain.

*Potentiation of noradrenaline effects on rat vas deferens* in vitro. Fluvoxamine was only weakly active in potentiating NA when compared with DMI and imipramine. Chlorimipramine produced only $\alpha$-lytic effects (Table 13).

*Antagonism of tetrabenazine-induced ptosis in mice.* Fluvoxamine was rather inactive in this test in comparison with the tricyclic antidepressants. The finding that imipramine was more active orally than intraperitoneally might be due to the fact that the metabolism of imipramine to the more active DMI occurs to a larger extent after oral than after intraperitoneal administration (Table 14).

**Table 12** Effect on noradrenaline release from rat brain by H 75/12 and H 77/77

| *Test compound (2 × 25 mg/kg i.m.)* | *Releasing agent* | *Brain noradrenaline content (μg/g tissue)* |
|---|---|---|
| Control | — | 0.34±0.009 (8) |
| Fluvoxamine | — | 0.34±0.027 (7) |
| Imipramine | — | 0.34±0.011 (6) |
| Desmethylimipramine | — | 0.33±0.022 (6) |
| — | H 75/12 | 0.18±0.008 (8) |
| Fluvoxamine | H 75/12 | 0.16±0.006 (8) |
| Imipramine | H 75/12 | 0.20±0.008 (8) |
| — | H 77/77 | 0.13±0.007 (8) |
| Fluvoxamine | H 77/77 | 0.14±0.006 (8) |
| Desmethylimipramine | H 77/77 | 0.28* ±0.008 (8) |

Values are the means ± s.e. mean; the number of determinations is given in parentheses. H 77/77 was injected at two doses of 12.5 mg/kg with a 2 h interval. The test compounds were injected intramuscularly 30 min before each H 77/77 injection. H 75/12 was injected using the same dosing schedule but with two doses of 25 mg/kg.

* *P* value <0.001 in comparison with appropriate control group (Student's *t* test).

**Table 13** Potentiation of effect of noradrenaline (NA) on rat vas deferens *in vitro*

| *Test compound* | *Minimal conc. causing potentiation (ng/ml)* | *Conc. causing optimal potentiation (ng/ml)* | *Degree of optimal potentiation* |
|---|---|---|---|
| Fluvoxamine | 200 (2) | 2000 (2) | 3.4 (4)<br>2.8–4.0 |
| Imipramine | . 7 (2) | 70 (2) | 3 8 (2)<br>3.4–4.4 |
| Desmethylimipramine | 2 (2) | 70 (2) | 19 (2)<br>12–31 |
| Chlorimipramine | only α-lytic effects | | |

Minimum concentration value indicates lowest concentration of test compound at which NA-induced contraction of vas deferens was increased. Optimal concentration value indicates concentration above which no further potentiation of the NA-induced contraction could be obtained In parentheses the number of experiments; for fluvoxamine in 2 experiments spontaneous contractions were observed at 2000 ng/ml. The minimal and optimal concentration were estimated by testing the test compounds in a concentration range of 2, 7, 20 etc. ng/ml in combination with a suprathreshold dose of NA Degree of optimal potentiation: apparent relative potency of NA when measured in presence of optimal concentration of test compound in comparison to NA without test compound in a series of four-point assays; 95% confidence limits indicated

*Antagonism of tetrabenazine-induced ptosis and induction of compulsive hyperactivity in rats.* Fluvoxamine had only a very weak activity against tetrabenazine-induced ptosis in the rat, in contrast to the tricyclic antidepressants (Table 15). Fluvoxamine was not active in inducing hyperactivity when administered at doses up to 215 mg/kg in combination with tetrabenazine. DMI and imipramine showed a clear activity, DMI being the most active compound in this respect. Chlorimipramine failed to induce compulsive hyperactivity at the highest dose tested, namely 46 mg/kg (Table 15).

**Table 14** Antagonism of tetrabenazine-induced ptosis in mice

| | *$ED_{50}$ (mg/kg)* | |
|---|---|---|
| *Test compound* | *Intraperitoneally administered* | *Orally administered* |
| Fluvoxamine | 35 ± 3.0 (2) | 107 ± 25 (9) |
| Chlorimipramine | 8.0 ± 1.0 (2) | 12 ± 2.7 (4) |
| Imipramine | 8.7 ± 1.0 (3) | 5.2 ± 0.3 (5) |
| Desmethyl-imipramine | 0.47 ± 0.17 (2) | 0.80 ± 0.09 (7) |

Values are the means ± s.e. mean; the number of determinations is given in parentheses Test compound was administered to groups of 5 mice in a range of doses intraperitoneally or orally, 45 min before a subcutaneous dose of 80 mg/kg of tetrabenazine. Ptosis was scored 45 min after tetrabenazine administration using a ptosis chart (Rubin, Malone, Waugh & Burke, 1957). $ED_{50}$ value, the dose of the test compound required to reduce the ptosis score to half that of the tetrabenazine control group, was obtained from the graph relating percentage response on a probit scale and log dose.

## Discussion

There is increasing support for the view that different subgroups of patients with depressive disorders may

**Table 15** Antagonism of tetrabenazine-induced ptosis and induction of hyperactivity in rats

| | *Oral $ED_{50}$ (mg/kg)* | |
|---|---|---|
| *Test compound* | *Ptosis antagonism* | *Hyperactivity* |
| Fluvoxamine | ≥215 (2) | >215 (3) |
| Chlorimipramine | 19 ± 2 4 (4) | >46 (4) |
| Imipramine | 4.0 ± 0.16 (16) | 15 ± 3.0 (2) |
| Desmethyl-imipramine | 5.1 ± 1.0 (7) | 8.3 (1) |

Values are the means ± s.e. mean; the number of determinations is given in parentheses. Test compound was administered to groups of 5 rats in a range of doses 45 min before a subcutaneous dose of 40 mg/kg of tetrabenazine. Ptosis was scored 90 min after tetrabenazine administration using a ptosis chart. $ED_{50}$ value, the dose of the test compound required to reduce the ptosis score to half that of the control tetrabenazine group, was obtained from the graph relating percentage response on a probit scale and log dose. The number of rats per dose group showing hyperactivity after tetrabenazine administration was scored. $ED_{50}$ value, the dose of test compound required to induce, in combination with tetrabenazine, hyperactivity in 50% of the animals, was calculated according to Horn (1956).

exhibit different specific abnormalities in the metabolism of biogenic amines. This may be the reason why various clinically defined subtypes of depression respond differently to antidepressant treatment (Hollister, 1972).

From recent investigations it is clear that the catecholamine hypothesis does not adequately explain the depressed state. For instance the catecholamine precursor L-DOPA has been a failure in therapy, except for a small subgroup of retarded depressives. Lapin & Oxenkrug (1969) suggested an important involvement of 5-HT in the pathogenesis of affective disorders. According to this view, activation of the central adrenergic functions is responsible for the psychoenergetic and motor-stimulating effects of antidepressants, whereas activation of serotoninergic functions is responsible for their mood-elevating properties. Confirmation of a possible role of a disturbance of 5-HT metabolism in depression comes from studies in which a decreased 5-HT content in the brain and a decreased 5-HT turnover has been demonstrated in patients (Shaw, Camps & Eccleston, 1967; van Praag, Korf & Puite, 1970; van Praag, Korf & Schut, 1973). It must be stated however that not all studies are in agreement concerning the occurrence of a reduced level of 5-HT metabolites in the cerebrospinal fluid of depressed patients (Papeschi & McClure, 1971).

Since the significance of 5-HT in the pathogenesis of affective disorders has not been clarified, there seems to be an urgent need for a drug with a specific effect on the 5-HT re-uptake processes, both as a specific therapeutic agent in suitable patients as well as a tool in classifying depressed patients. Fluvoxamine promises to be a member of such a new class of drugs.

The marked activity of fluvoxamine as a 5-HT uptake inhibitor was demonstrated in a number of test systems. Blood platelets may be used as a model for storage-, release- and uptake-processes of 5-HT in the nerve ending. Todrick & Tait (1969) compared the inhibitory properties of a large series of tricyclic antidepressants on 5-HT uptake. They found that the tertiary amines were more potent inhibitors than their demethylated derivatives; this is in accordance with the *in vivo* results of Carlsson, Fuxe & Ungerstedt (1968). In our experiments, the inhibitory activity of fluvoxamine on 5-HT uptake by blood platelets was found to be comparable to the activity of imipramine (Table 1). Chlorimipramine appeared to be clearly more active, a result also obtained by Todrick *et al.* (1969). However, such a superior activity for chlorimipramine, was not found in a recent investigation by Tuomisto (1974) who studied the effects at very low substrate concentrations and with very short incubation times. Although the experiments of Tuomisto may be criticized on the basis of a badly controlled incubation temperature, this result of a comparable activity of imipramine and chlorimipramine would agree better with the results of our *in vivo* experiments, in which for chlorimipramine no outstanding 5-HT potentiating properties were found.

The 5-HT uptake inhibiting properties of fluvoxamine, imipramine and DMI were compared in studies with rat brain synaptosomes (Tables 2–4). All three compounds showed a competitive inhibition of the uptake process. Under both *in vitro* conditions and in studies in which synaptosomes from animals pretreated with the test compounds were used, it was apparent that fluvoxamine produced a markedly stronger inhibition of 5-HT uptake than imipramine. Imipramine in its turn was more active than DMI.

An indirect indication of the effectiveness of fluvoxamine in inhibiting the uptake mechanism of serotoninergic neurones was obtained in the *in vivo* experiments with H 75/12 and H 77/77. These tyramine derivatives utilize the neuronal membrane pump to enter the neurone, where they cause a depletion of 5-HT. Here a marked inhibitory activity of fluvoxamine was apparent (Table 5). Imipramine also showed a protective effect at the same dose level although to a lesser extent than did fluvoxamine; DMI was only weakly active. The change in turnover rate of the neurotransmitter *in vivo* after administration of the test compound is generally used as an indication of the change in the neuronal activity of that aminergic system. A direct or an indirect increase of the activity of postsynaptic fibres produces a negative feedback in the presynaptic system, which expresses itself by a lowering of the turnover rate of the transmitter. Both after intramuscular as well as after oral administration, fluvoxamine produced a marked decrease in 5-HT turnover rate as measured by the probenecid test (Table 6). Against the background of the effects of fluvoxamine shown in the various experiments, it may be concluded that this decrease of the turnover rate of 5-HT probably results from the inhibition of the re-uptake of 5-HT from the synaptic cleft by the presynaptic neurone. Imipramine showed the same effect on 5-HT turnover rate, but possibly to a lesser extent.

Further evidence for the prominent activity of fluvoxamine as a 5-HT uptake inhibitor was obtained in mice by the potentiation of the induction of behavioural effects by 5-HTP (Table 7). These 5-HTP-induced effects are probably due to the formation of an increased amount of 5-HT in the brain, since we found that these signs are suppressed by centrally acting anti-5-HT compounds and also by high doses of centrally acting decarboxylase inhibitors. In comparison with the tricyclic antidepressants, fluvoxamine possesses outstanding oral activity. This is demonstrated to an even greater extent by the induction of 5-HT-like effects in mice, pretreated with a MAO-inhibitor. All the tested tricyclic antidepressants were almost inactive in this test (Table 8).

Further suggestive evidence, for a potentiation of effects of 5-HT by fluvoxamine, was obtained from the experiments on the antagonism of the reserpine-induced lowering of the pentamethylenetetrazole convulsive threshold (Table 9). Although most reserpine effects which are used for demonstrating antidepressant activity are dependent upon NA-mechanisms, the convulsion-facilitating effect may be attributable to the depletion of 5-HT (Boggan, 1973). In this test fluvoxamine also showed the highest activity; of the tricyclic antidepressants tested, only chlorimipramine was active. These results therefore support the hypothesis of Boggan (1973).

Whereas tricyclic antidepressants are characterized by a pronounced inhibition of the NA-uptake process, fluvoxamine in general demonstrated only a very weak NA-uptake inhibiting activity. A comparable, rather weak, uptake inhibiting activity was noted for fluvoxamine and DMI in experiments in which the uptake of labelled NA was measured. However, the use of total brain synaptosomes in this way does not differentiate very well between activities of NA uptake inhibitors (Schacht & Heptner, 1974). Fluvoxamine produced only a marginal NA-uptake inhibiting effect when the compound was intraperitoneally administered to the animal in a dose of 25 mg/kg (Table 11). Also in experiments in which the displacement of labelled NA from the synaptosomes by unlabelled amine in the incubation medium was studied, fluvoxamine was again much less active than the tricyclic antidepressant drugs (Table 10). The same ineffectiveness is apparent from the fact that in the rat, no protection was produced by fluvoxamine against the NA-depleting action of H 75/12 and H 77/77 (Table 12).

The NA effect on the rat vas deferens was potentiated by fluvoxamine only at much higher concentrations than those at which tricyclic antidepressants were active (Table 13).

Fluvoxamine is only weakly active in antagonizing reserpine-like effects; this is again in contrast to the tricyclic antidepressants (Tables 14–17). For example, fluvoxamine possesses an $ED_{50}$ value of 107 mg/kg in antagonizing tetrabenazine-induced ptosis in mice and thus is 9 to 130 times less active than the tested tricyclic drugs. In antagonizing tetrabenazine-induced ptosis in rats, fluvoxamine was even less active. Experiments, not presented, have also shown that reserpine-induced hypothermia in mice was reversed by fluvoxamine at much higher dose levels than those at which tricyclic antidepressants showed a significant effect. In contrast to most tricyclic antidepressants fluvoxamine did not produce compulsive hyperactivity in the rat when administered in combination with tetrabenazine (Table 15).

Since reserpine-induced effects can be antagonized by monoamine oxidase (MAO) inhibitors, experiments were performed to show that fluvoxamine did not inhibit this enzyme. Fluvoxamine, at concentrations up to $10^{-3}$ M, did not cause significant inhibition of MAO using kynuramine and 5-HT as substrates and ox brain and liver as enzyme sources. In contrast to MAO inhibitors, fluvoxamine, at an oral dose of 460 mg/kg in mice, failed to potentiate the toxicity due to tryptamine (250 mg/kg s.c.) administered either 1 or 24 h later. This lack of *in vivo* MAO inhibitory activity was also confirmed by the finding that the compound did not cause a significant change in the NA, dopamine or 5-HT contents of rat brain or heart after daily dosing during either 7 or 35 days at a dose of 100 mg/kg per day.

Based on the biochemical and pharmacological studies reported here, fluvoxamine can be characterized as a potential anti-depressant drug with almost exclusively 5-HT-uptake inhibiting properties and the compound should therefore offer better possibilities for the treatment of depression in cases where disturbances of 5-HT metabolism are prominent.

The role of 5-HT in a number of physiological processes and possible deficiencies of serotoninergic functions in various pathological conditions seem to indicate that fluvoxamine should be tested in a number of additional disease states.

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# TAMOXIFEN INHIBITION OF AN *in vitro* OESTRADIOL-INDUCED SURFACE COAT CHANGE ON MOUSE BLASTOCYSTS

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1 Eighty-nine hour post-coitus mouse blastocysts cultured in the presence of oestradiol-17β underwent a histochemically detectable surface coat change.

2 Tamoxifen was found to inhibit this *in vitro* -induced change. The role of Tamoxifen as an inhibitor of implantation is discussed.

## Introduction

Tamoxifen (ICI 46,474, *trans* 1(*p*-(2-dimethylamino-ethoxy)-phenyl)-1,2-diphenylbut-1-ene) has been shown previously to prevent implantation of the blastocyst in rats and mice (Harper & Walpole, 1967a, b) and to prevent *in vivo* the oestrogen-dependent surface coat change of the late preimplantation mouse blastocyst (Bloxham, Pugh & Sharma, 1975). These authors pointed out that the latter effect could arise directly, from an inhibition of the uptake of oestradiol by the blastocyst, or indirectly, following interference with the synthesis, release or fate of the post-ovulatory oestrogen surge in the mother.

In order to elucidate further the mode of action of the drug in mice we have studied its effects on the oestrogen-induced surface coat change of the mouse trophoblast *in vitro*.

## Methods

Albino random-bred mice were maintained in a chamber with a standardized 10 h dark period centred on 17 h 00 min. In determining the precise time for killing the mice, so as to collect ova of a known age, mating was considered to have taken place at 17 h 00 min on day zero.

The mated mice were randomly divided into three groups. Group I (4 mice) were killed at 89 h post-coitum (h p.c.) and group II (5 mice) were killed at 96 h post-coitum. The blastocysts were collected and fixed for 24 h in 10% neutral buffered formalin, before being air-dried onto slides and stained according to the method of Holmes & Dickson (1973), as used in this laboratory (Bloxham *et al.*, 1975). This technique stains the blastocyst trophoblast red, before, or blue, after, the surface coat change. Group III (26 mice) were killed at 89 h p.c., their blastocysts were collected in Whittingham (1971) culture medium, washed in fresh medium and randomly allocated into ten sub-groups of 25 blastocysts each. The sub-groups were placed in embryological watch glasses containing either 2 ml culture medium (Whittingham, 1971) or medium to which oestradiol-17β (Sigma) or Tamoxifen (ICI), or both, at various concentrations were added (Table 1) as follows. Both the oestradiol and Tamoxifen were made up in absolute ethanol and the amount appropriate to the desired concentration in the culture medium was in each case pipetted into a test tube and dried down; 2 ml of culture medium was added and the tube was vortex mixed for 30 s before tipping the contents into the watch glass. The blastocysts were cultured in an incubator at 37 ± 0.5°C, for 16 h, after which time they were removed and washed in phosphate-buffered normal saline before fixation and staining as described above.

## Results

All group I (89 h p.c.) mouse blastocysts (38) were red after staining while all the group II (96 h p.c.) blastocysts (53) stained blue, so reconfirming the occurrence of the trophoblast surface coat change between 89 and 96 h p.c. *in vivo*.

The group III 89 h p.c. blastocysts cultured in Whittingham medium (sub-group a) failed to undergo the trophoblastic surface coat change even by 105 h post coitum. The presence of 25 pg/ml or more oestradiol in the culture medium (sub-groups b and c) had enabled the surface coat change to take place by 105 h p.c. in all the blastocysts so treated. Tamoxifen alone, on the other hand, added to give a concentration of 100 pg/ml culture medium did not enable the coat change to occur (sub-group f). This concentration of Tamoxifen was also able to prevent the anticipated change in the surface coat in the presence of 50 pg/ml oestradiol-17$\beta$ (sub-group g). Progressive reduction of the amount of Tamoxifen added to successive sub-groups in the presence of 50 pg/ml oestradiol-17$\beta$ allowed an increasing percentage of the eggs to undergo the surface coat change (sub-groups h, i and j). The composition of the group III sub-groups and the percentage occurrence of the trophoblastic surface coat change in the individual groups of 25 blastocysts are detailed in Table 1.

## Discussion

Dickmann, Dey & Sen Gupta (1975) have suggested on the basis of indirect evidence that oestradiol, perhaps originating from the pre-implantation embryo itself, is necessary for the maturation and later implantation of the embryo.

Wilson & Jenkinson (1974) reported that when maintained *in vitro* in simple buffered salt solutions (BSS) containing albumin, development of the embryo ceases at the expanded blastocyst stage and that changes analogous to those of implantation are seen only when otherwise chemically defined culture media are enriched, e.g. with 30% foetal calf serum.

The experiments described in this paper confirm the limitations of culture in BSS + albumin but show that the addition of oestradiol-17$\beta$ to the fully chemically defined medium allows maturation to progress further in that the pre-implantation surface coat change is enabled to occur. This change is believed to reflect the secretion of acidic glycoproteins by the trophoblastic cells (Pinsker & Mintz, 1973) and our evidence of the occurrence of the coat change *in vitro* would appear to support this possibility, rather than an origin somewhere in the uterus. The necessity for oestradiol-17$\beta$ for implantation in the mouse is well established (Smith & Biggers, 1968; McLaren, 1971; Holmes & Dickson, 1973). The results of this study raise the possibility that induction of the surface coat change is one mechanism by which oestradiol-17$\beta$ could act in bringing about implantation. The need for a major change in surface coat properties before the adhesion phase of implantation was postulated by Enders & Schlafke (1974).

Tamoxifen is established as an antifertility agent in the mouse, acting by preventing implantation and has been shown to exert antioestrogenic effects (Harper & Walpole, 1967a). The compound competes with oestradiol-17$\beta$ for receptors in mouse uterus cytosol (Skidmore, Walpole & Woodburn, 1972) and related drugs decrease the rate of replenishment of cytosol receptors (Clark, Peck & Anderson, 1976). Having already demonstrated that Tamoxifen in preventing implantation in the mouse also prevents the surface coat change *in vivo* (Bloxham *et al.*, 1975), it was of interest to examine its effect on the oestradiol-induced surface coat change under *in vitro* conditions so as to characterize further the anti-implantation action of the drug. In that Tamoxifen was shown to inhibit the *in vitro*, oestradiol-17$\beta$-induced, surface coat change in a concentration-dependent manner, it now seems reasonable to suggest that this antioestrogenic action could contribute to the antifertility effect of the drug *in vivo*.

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**Table 1** Drug concentrations in the 2 ml of culture medium in which 25 mouse blastocysts were maintained for 16 hours.

| *Sub-group* | *Oestradiol -17β (pg/ml)* | *Tamoxifen (pg/ml)* | *% blastocysts with surface coat change* |
|---|---|---|---|
| (a) | 0 | 0 | 0 |
| (b) | 50 | 0 | 100 |
| (c) | 25 | 0 | 100 |
| (d) | 12.5 | 0 | 63.7 |
| (e) | 6.25 | 0 | 0 |
| (f) | 0 | 100 | 0 |
| (g) | 50 | 100 | 0 |
| (h) | 50 | 50 | 16 6 |
| (i) | 50 | 25 | 88 |
| (j) | 50 | 12.5 | 100 |

The percentage occurrence of the surface coat change within each group at this time, as revealed histochemically, is entered in the final column.

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# OLFACTORY BULB ABLATION IN THE RAT: BEHAVIOURAL CHANGES AND THEIR REVERSAL BY ANTIDEPRESSANT DRUGS

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1 The effects of bilateral olfactory bulbectomy, sham-operation and inducement of peripheral anosmia were studied on locomotor activity, passive avoidance acquisition and irritability.

2 Bulbectomized rats were hyperactive, deficient at learning a step-down passive avoidance response and hyperirritable. Peripheral anosmia, induced by intranasal infusion of $ZnSO_4$ solution resulted in no behavioural changes.

3 Chronic pretreatment with amitriptyline (3 and 10 mg/kg) and a tetracyclic antidepressant mianserin (Org GB 94, 5 and 15 mg/kg) reversed the hyperactivity and reduced the learning deficit of bulbectomized rats. These drugs had no significant effects on sham-operated animals.

4 Neither amitriptyline nor mianserin reduced the exaggerated responses of bulbectomized rats to external stimuli.

5 (+)-Amphetamine (1 and 3 mg/kg) accelerated the acquisition of the passive avoidance response, greatly enhanced the locomotor activity and slightly increased the irritability score of both sham-operated and bulbectomized rats.

6 Chlorpromazine (1 and 3 mg/kg) and chlordiazepoxide (10 mg/kg) significantly reduced the acquisition, locomotor activity and irritability of experimental and control rats.

7 Lithium sulphate (1 and 3 mg/kg) had no effect on activity or irritability but produced a small impairment in acquisition of bulbectomized rats.

8 It is concluded that the reversal by antidepressant drugs of the behavioural syndrome seen after olfactory bulb ablation could constitute a new model for the detection of this group of centrally acting compounds.

## Introduction

Established tests for the prediction of antidepressant activity often give either false positives (Zetler, 1963; Barnett, Taber & Roth, 1969) or false negatives (van Riezen, Behagel & Chafik, 1975). Mianserin (Org GB 94) is an example of a drug which was not classified as having antidepressant activity by the usual tests (van Riezen, 1972). However, period analysis of alert electroencephalograms in human volunteers revealed activity similar to that of amitriptyline (Itil, Polvan & Hsu, 1972) and subsequent clinical experience has shown mianserin to be an effective antidepressant (Murphy, 1975; Wheatley, 1975).

In 1971 Cairncross & King reported that behavioural deficits resulting from bilateral ablation of the olfactory bulbs of rats were reversed by chronic treatment with amitriptyline. This suggested that removal of the olfactory bulbs may produce a suitable model for the screening of antidepressant drugs. We have recently reported that both amitriptyline and mianserin reversed the deficit in conditioned behaviour and reduced the hyperactivity of bulbectomized rats (van Riezen, Schnieden & Wren, 1976). We have now enlarged the battery of tests in order to characterize further the 'syndrome' seen after olfactory bulbectomy, and challenged the model with other psychotropic agents.

## Methods

### Animals and operations

Male Sprague-Dawley rats weighing between 175–215 g were used throughout the experiments. The rats were anaesthetized with sodium pentobarbitone (40 mg/kg, i.p.). A longitudinal incision was made in the midline, 2 cm long and caudal to the rim of the orbit. Burr holes 2 mm in diameter were drilled through both frontal bones, 2 mm lateral to the frontal suture at a point level with the posterior rim of the orbit. The underlying olfactory bulbs were aspirated using a round ended needle 1.5 mm in diameter. Care was taken to avoid damaging the frontal cortex. The holes were plugged with gel foam (Spongostan Film, 200 × 70 × 0.5 mm; Ferrosan, Denmark) and the wound sutured.

Sham-operated rats were subjected to a procedure identical to that described above, except that immediately after exposure of the olfactory bulbs, the burr holes were plugged and the skin incision sutured.

Upon recovery the rats were housed 5 to a cage, each cage containing both bulbectomized and sham-operated rats. Drug pre-treatment began on the fourteenth day after surgery and behavioural testing on day 21.

Peripheral anosmia was produced by a method similar to that of Alberts & Galef (1971). A hooked catheter was inserted into the mouth of a rat anaesthetized with ether with the bent tip of the catheter facing downwards. It was run back along the hard palate until the rounded apex was felt to enter the oesophagus. The catheter was retracted rostrally so that the tip entered the nasal cavity via the posterior choanae. Five percent (w/v) $ZnSO_4$ solution or saline of similar osmolality (0.5–0.7 ml) was slowly injected until eight drops drained from the external nares. Twenty-four hours following treatment the ability of the rat to detect olfactory cues was tested, before subjecting them to the battery of behavioural experiments.

### *Test for anosmia*

Five small pieces of dried rodent food (Oakes, P.M.D.) sweetened with evaporated milk were placed on the floor of a clean cage, measuring 20 × 40 cm. They were covered with 2.5 cm clean wood shavings. Each rat in turn was placed in the cage and left there for 15 min or until it had located all of the food pellets. The cage was cleaned and fresh shavings and impregnated pellets used for each test.

### *Locomotor activity test*

Locomotor activity was measured with an Animex meter (LKB Farad, Sweden), sensitivity and tuning: 40 µA. Rats were placed individually in a clean perspex cage without bedding material and having a floor area of 1000 cm². The rat had access to food and water. The cage was placed on the centre of the meter which was in a darkened room. Activity counts were recorded at 5 min intervals for 30 minutes.

### *Passive avoidance experiment*

The apparatus consisted of an open-topped perspex box 55 cm square with a stainless steel grid floor. The rods were 1.2 cm apart and connected to the terminals of a stimulator (SRI Model 6051) delivering square wave pulses at a constant voltage through a 'scrambler' unit. The shock delivered was constant at 0.85 mA (70 V, 6 ms, 6 Hz).

The rat's paws were wiped with a damp cloth to improve electrical conductivity and it was placed on the central wooden platform, 19 cm square and 4 cm above grid level. The latency time for the rat to step off the platform with all four paws was measured. The rat was immediately removed and the procedure repeated with an intertrial interval of 30 s until the rat remained on the platform for two minutes. The rat was then considered to have learnt to avoid the foot-shock. The number of trials needed by each rat to reach this learning criterion was recorded.

### *Irritability scoring test*

The 'irritability' of a rat was scored by means of an arbitrary numerical system similar to that of King (1958) and Nurimoto, Ogawa & Ueki (1974) (Table 1). The stimuli used were: (a) a puff of air blown sharply on to the back of the rat whilst it was facing away from the experimenter; (b) a loud handclap delivered close to and in front of the rat's nose.

### *Estimation of pain threshold*

Immediately before the passive avoidance experiment each rat was placed on the grid floor of this apparatus and footshock applied for periods of 0.7 s, increasing the intensity by 0.05 mA every 30 s until the animal showed visible signs of discomfort (flinching). The current intensity at this point was used to provide an estimate of pain threshold.

**Table 1** Scoring system (after King, 1958) used for irritability scoring test

| *Score* | *Response* |
|---|---|
| 0 | No response |
| 1 | Slight—rat raises head/ moves away from stimulus |
| 2 | Moderate—rat jumps and freezes for 0–2 s |
| 3 | Marked—rat jumps and freezes for 2–15 s |
| 4 | Extreme—rat jumps and freezes for > 15 s |

The maximum score attainable for two stimuli was eight.

### *Statistics*

The results of the locomotor activity and passive avoidance experiments were analysed using a two-tailed Student's *t* test. Significance values of $P<0.05$ were used except where otherwise stated. The significance of the results of the irritability scoring test was determined by a Mann Whitney U test.

## Results

### *Verification of lesions*

In these studies olfactory bulbectomy was assessed macroscopically. All rats in which less than 70% of the bulbs had been removed or whose frontal cortex had been damaged were omitted from the results. In every successful bulbectomy the accessory olfactory bulbs had also been ablated.

### *Anosmia testing*

All five hypotonic saline-treated rats found and ate the pellets within the 15 min period. The rats receiving an intranasal injection of $ZnSO_4$ solution all failed to find any pellets although they explored the cage thoroughly.

### *Pain threshold*

None of the experimental groups was significantly different from controls with respect to the intensity of footshock required to elicit a pain response (see Table 2).

**Table 2** Effects of bulbectomy (OB), $ZnSO_4$ application and drugs on pain threshold

| *Treatment* | n | *Mean threshold footshock intensity (mA ± s.e.)* |
|---|---|---|
| Controls* | 31 | 0.45 ± 0.02 |
| OB | 26 | 0.44 ± 0.02 |
| $ZnSO_4$ | 5 | 0.47 ± 0.01 |
| OB + Ami (10 mg/kg) | 5 | 0.45 ± 0 02 |
| OB + M (15 mg/kg) | 5 | 0.48 ± 0.02 |

$n$ = number of rats per group

* This group includes both sham-operated rats and those treated with intranasal infusion of hypotonic saline. OB rats were pretreated with either amitriptyline (Ami) or mianserin (M) for seven days before testing.

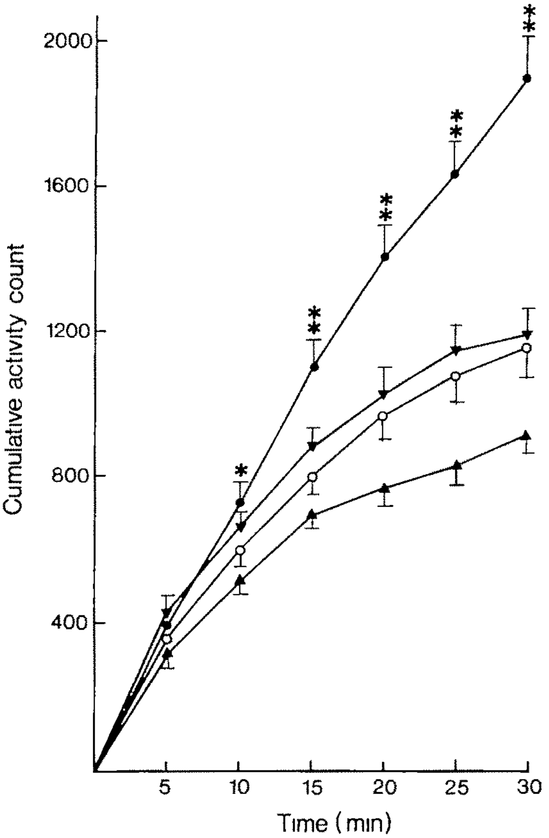

**Figure 1** The effects of centrally or peripherally induced anosmia on the locomotor activity of rats. Fourteen days after surgery or 24 h after $ZnSO_4$ or hypotonic saline infusion, rats were placed on an Animex meter for a single 30 min session. (○) Sham-operation, $n=26$; (▲) hypotonic saline infusion, $n=5$, (▼) $ZnSO_4$ infusion, $n=5$; (●) bulbectomy, $n=26$ Points represent mean activity and the vertical bars indicate s e. mean.

* Significantly different from sham-operated controls $P<0.05$, **$P<0.001$ as determined by Student's *t* test (two-tailed).

### *Spontaneous locomotor activity*

Bulbectomized rats had a significantly higher activity throughout the 30 min recording period than sham-operated animals (Figure 1). There were no significant differences between the activity of the control rats and those treated with $ZnSO_4$. Both amitriptyline and mianserin brought about a dose-dependent decrease in the activity of the bulbectomized rats, the decrease due to the higher dose being significantly different ($P<0.05$) from that of the lower dose as well as from placebo-treated control activity ($P<0.001$, Figure 2a, b), but had no significant effect on the sham-operated animals (Figure 2c, d).

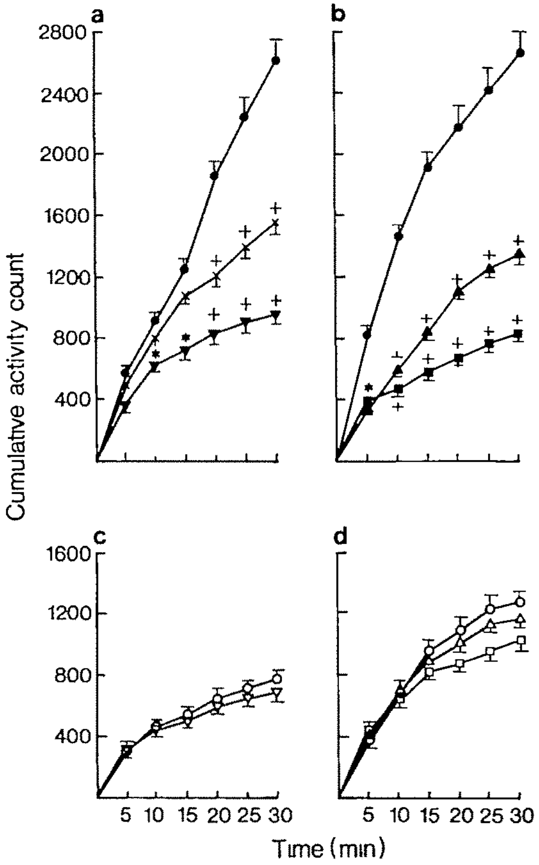

**Figure 2** Locomotor activity *v* time curves for (a) bulbectomized rats pretreated with amitriptyline; (b) bulbectomized rats pretreated with mianserin; (c) sham-operated rats pretreated with amitriptyline; (d) sham-operated rats pretreated with mianserin. Chronic drug pretreatment began 14 days after surgery and locomotor activity using an Animex meter was measured on day 21. Closed symbols represent mean values for bulbectomized rats, open symbols for sham-operated rats. (●, ○) Saline pretreatment, $n=8$; (x) amitriptyline 3 mg/kg, $n=5$; (▼, ▽) amitriptyline 10 mg/kg, $n=5$; (▲, △) mianserin 5 mg/kg, $n=5$; (■, □) mianserin 15 mg/kg, $n=5$. Vertical bars indicate s.e. mean.

* and $^{+}$ Significantly different from respective saline-pretreated controls ($P<0.05$ and $P<0.001$) as determined by Student's *t* test (two-tailed).

Amphetamine produced a significant dose-dependent increase in the acitivity of both bulbectomized and sham-operated groups whereas chlorpromazine and chlordiazepoxide brought about a similarly significant reduction in locomotor activity. Pretreatment with lithium sulphate resulted in no significant changes (see Figure 3).

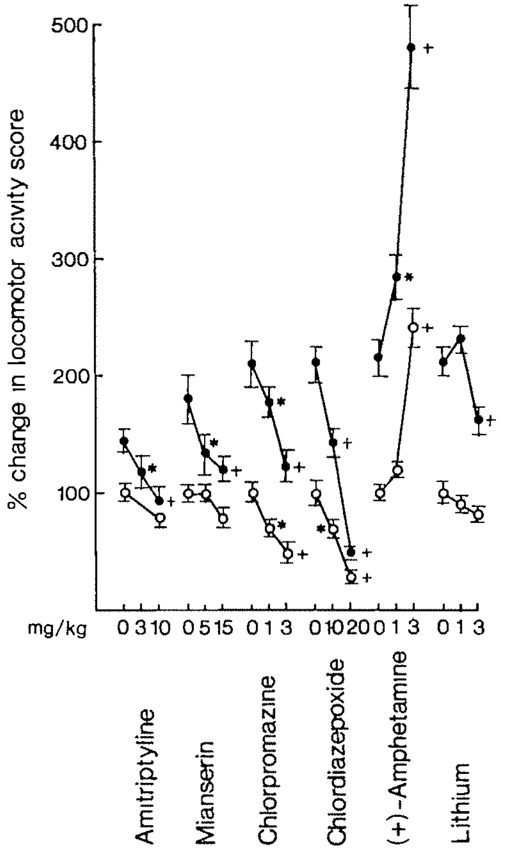

**Figure 3** Percentage change in locomotor activity scores (locomotor activity score of placebo-pretreated sham-operated rats = 100%) for bulbectomized (●) and sham-operated (○) rats after pretreatment with psychotropic drugs.

Chronic drug pretreatment began 14 days after surgery and locomotor activity was measured, using an Animex meter, on day 21. Points represent the mean values of between 5 and 8 rats. Vertical bars indicate s.e. mean.

* and $^{+}$ Significant differences ($P<0.05$ and $P<0.001$; two-tailed Student's *t* test) from respective saline-pretreated controls.

### *Passive avoidance acquisition*

The results of this experiment are shown in Figure 4. Bulbectomized rats required a significantly greater number of trials to acquire the response than either sham-operated or $ZnSO_4$-treated rats. The performance of bulbectomized rats pretreated with either amitriptyline, or mianserin was significantly improved in a dose-dependent manner but similarly treated sham-operated rats showed no greater tendency to avoid the footshock.

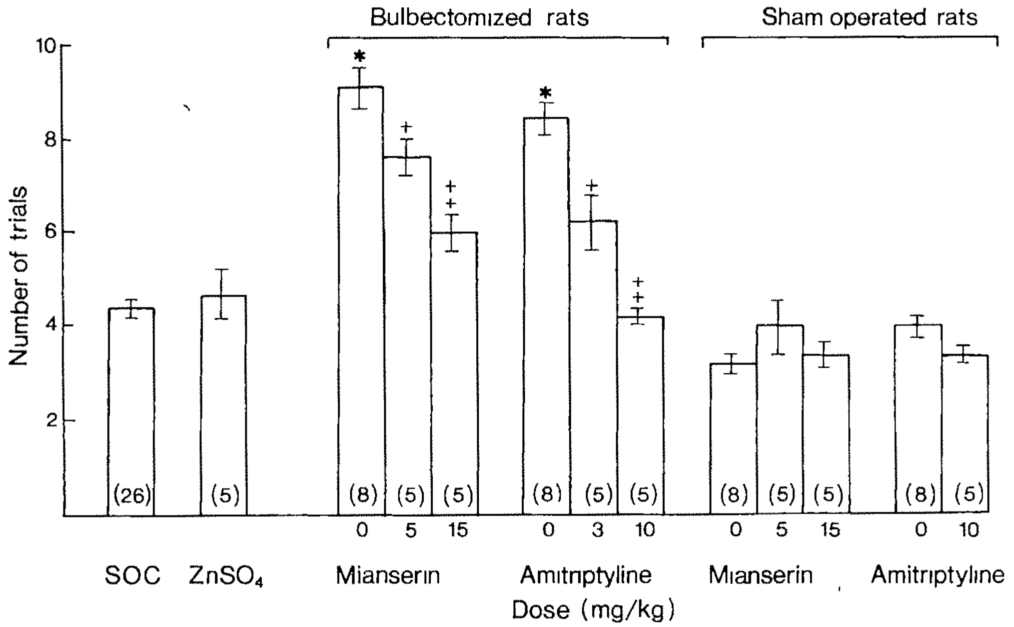

**Figure 4** The effects of bulbectomy, $ZnSO_4$ treatment and drug pretreatment on passive avoidance acquisition. Chronic drug pretreatment began 14 days after surgery and the passive avoidance test was carried out on day 21. Each column represents the mean number of trials needed by each group to acquire the response (avoidance of footshock by remaining on platform for at least two minutes). S.O C. = sham-operated controls Numbers in parentheses at base of columns show the group size Vertical bars indicate s e. mean. * Significantly different from sham-operated controls ($P < 0.001$), + and ‡ significantly different from saline-pretreated bulbectomized rats ($P < 0.05$ and $P < 0.001$ respectively) as determined by Student's $t$ test (two-tailed).

**Table 3** Effects of drug pretreatment on passive avoidance and irritability

| | | *Step-down passive avoidance*[1] | | *Irritability*[2] | |
|---|---|---|---|---|---|
| *Drug* | *mg/kg* | *Bulbectomized rats* | *Sham-operated rats* | *Bulbectomized rats* | *Sham-operated rats* |
| Amitriptyline | 3 | – – | / | 0 | / |
| | 10 | – – – | 0 | – | 0 |
| Mianserin | 5 | – – | 0 | 0 | 0 |
| | 15 | – – – | 0 | – | 0 |
| Amphetamine | 1 | – – – | – – – | 0 | 0 |
| | 3 | – – – | – – | + | + + |
| Chlorpromazine | 1 | 0 | + + | – | 0 |
| | 3 | + + + | + + + | – – – | – |
| Chlordiazepoxide | 10 | + + | + + | – – | – |
| Lithium | 1 | 0 | 0 | 0 | 0 |
| | 3 | + + | 0 | 0 | 0 |

[1] Measured as the number of trials a rat required before acquiring the appropriate response, i.e. remaining on the safety platform for at least 2 minutes.

[2] Measured as the cumulative score obtained by grading the rat's response to two novel stimuli (for further details see text).

Rats were pretreated for 7 days before testing. Symbols represent the mean response of between 5 and 8 rats. (/) no experiment; (0) no effect; (– or +) decrease or increase in the number of trials or irritability score, $P < 0.1$–$0.05$; (– – or + +) $P < 0.05$–$0.01$; (– – – or + + +) $P < 0.01$–$0.001$.

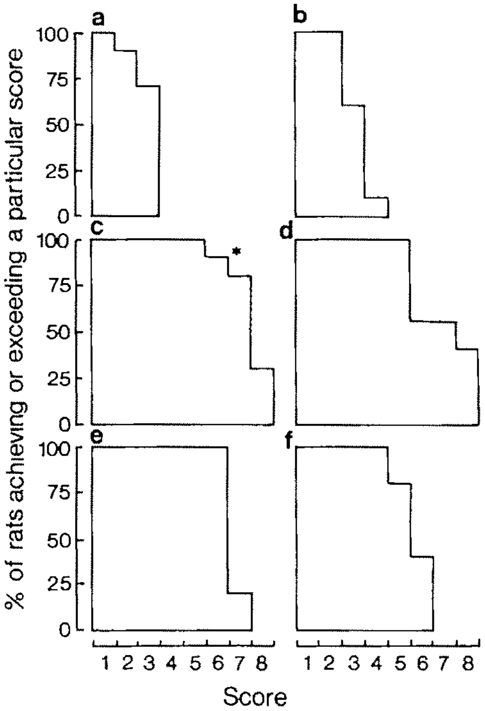

**Figure 5** Effects on the irritability score of (a) sham-operation and hypotonic saline infusion; (b) intranasal ZnSO$_4$ infusion; (C) bulbectomy; (d) saline pretreatment of bulbectomized rats; (e) chronic pretreatment of bulbectomized rats with amitriptyline (10 mg/kg); (f) chronic pretreatment of bulbectomized rats with mianserin (15 mg/kg). Numbers in parentheses show group size.
*Significantly different from sham-operated controls ($P<0.01$) as determined by a Mann Whitney U test. Drug pretreatment produced no significant changes.

Table 3 summarizes the results of this experiment. In comparison to the antidepressants, amphetamine improved the performance of both sham-operated and bulbectomized rats. Chlorpromazine and chlordiazepoxide significantly delayed acquisition in both groups while lithium sulphate slightly increased the deficit exhibited by bulbectomized rats.

*Irritability scoring*

The results of this experiment are shown in Figure 5. Most of the sham-operated, hypotonic-saline-treated and ZnSO$_4$-treated rats received a cumulative rating of 3 and none more than 4, whereas all bulbectomized animals scored at least 5 and 30% obtained the maximum possible score of 8. In addition to the exaggerated startle and freezing response, the bulbectomized rats would often attack the stimulus source. Both antidepressants slightly but non-significantly reduced the hyperirritability score of the bulbectomized rats. No change in the responses of the sham-operated rats pretreated with either amitriptyline or mianserin was observed.

Amphetamine produced a non-significant increase in the scoring. In contrast, pretreatment with either chlorpromazine or chlordiazepoxide resulted in a signficant reduction in the aggressive response of the bulbectomized rats. These drugs did not change the low scores of the sham-operated animals. Lithium again had no effect (see Table 3).

## Discussion

In agreement with Marks, Remley, Seago & Hastings (1971), Sieck (1972) and Cain (1974), we have demonstrated that removal of the olfactory bulbs precipitates a specific behavioural syndrome. One possibility for the behavioural changes was that they could be attributed to loss of olfactory sensibility. However, our experiments have shown that the performance of rats rendered peripherally anosmic was not significantly different from controls. Therefore this adds to the considerable number of reports suggesting that the alterations in behaviour observed after ablation of the olfactory bulbs are not due to anosmia *per se* (Hankins, Garcia & Rusiniak, 1973; Cain & Paxinos, 1974).

Examination of the locomotor activity results showed that the graph of the cumulative activity of the bulbectomized rats followed a straight line whereas that of sham-operated animals levelled off after 15–20 min (see Figure 1). Similar results have been reported (for both increased locomotor activity and passive avoidance deficits) for lesions in various limbic sites, for example, amygdala (Pellegrino, 1968), hippocampus (Douglas & Isaacson, 1966; McCleary, 1966), and septum (Thomas, 1972). These have been explained by a failure in the habituation of exploratory behaviour and a similar argument could be put forward for the bulbectomized rat, especially as the olfactory bulb has been shown to have a wealth of interconnections with the limbic system (Powell, Cowan & Raisman, 1965).

Because of the changes in affective and exploratory postlesion behaviour, some influence of olfactory bulb lesions on avoidance behaviour would be expected. We have confirmed this for the passive avoidance situation where bulbectomized rats are deficient at acquiring the response (Marks *et al.*, 1971; Sieck, 1972). In contrast, workers investigating the behaviour of such rats in two-way active avoidance experiments have reported faster escape and better avoidance learning (Brown, Harrell & Remley, 1971; Sieck & Gordon, 1972; Sieck, 1972; 1973). Most rats which were slow to reach the passive avoidance learning criterion were observed to adopt fixed 'escape' tendencies such as leaping for the cage top or jumping on to the grid as soon as they were placed on the platform. This suggests that bulbectomized rats appreciated that they would be shocked but were unable to acquire an appropriate avoidance response or were less able to inhibit the step-down response. The opposite effects on active and passive avoidance tests also argue against an explanation in terms of altered sensitivity to painful stimuli and, more directly, our results show that bulbectomized rats have similar pain thresholds to controls.

Before entering into a full discussion of the specific effects of the antidepressants amitriptyline and mianserin on the olfactory bulbectomy syndrome, it is constructive to consider the actions of the other psychoactive drugs used in this study. It can be seen that the major tranquillizer chlorpromazine and the anxiolytic drug chlordiazepoxide and lithium sulphate (at the higher dose) all exaggerated the learning deficit exhibited by bulbectomized rats in the step-down passive avoidance test. In contrast, amphetamine enhanced their performance in a manner similar to that of the antidepressants. Such an action of amphetamine is not surprising in view of its well-documented central effects. However, the behavioural effects of amphetamine can be differentiated from those of the antidepresants by examination of the locomotor activity results, when a clear difference emerges.

It is suggested therefore that the bulbectomized rat model dissociates the antidepressants from other classes of psychotropic drugs using tests measuring locomotor activity and passive avoidance responding. Amitriptyline and mianserin reversed both the increased activity and poor performance in the passive avoidance test exhibited by the bulbectomized rats, without lessening their irritability, as measured by grading their responses to sudden novel stimuli directed towards them. This suggests that bulbectomy produces two distinct deficits characterized by their sensitivity to antidepressant drugs. However, several workers have reported that bulbectomy facilitates mouse killing (Bugbee & Eichelman, 1972; Ueki, Nurimoto & Ogawa, 1972a). Ueki and co-workers (1972b) have demonstrated that amitriptyline selectively antagonizes this component of the 'hyper-irritability' syndrome. Caution should therefore be exercised when defining 'irritability' in future studies of bulbectomized animals, especially since in our experiments the pain thresholds of bulbectomized rats were not altered.

At the biochemical level Pohorecky, Zigmond, Heimer & Wurtman (1969) and Cairncross & King (1971) showed that bulbectomy results in a significant reduction in telencephalic noradrenaline. Subsequent chronic pretreatment with amitriptyline resulted in an enhancement of learning which was paralleled by an increase in the noradrenaline content of the telencephalon (Cairncross, Schofield & King, 1974). They suggested that the results offer a possible explanation for the mode of action of amitriptyline in the clinical situation. However Leonard (1974) concluded from neurochemical studies that, in contrast to the tricyclic antidepressants, mianserin does not affect the release or uptake of 5-hydroxytryptamine or noradrenaline after acute administration. Given chronically for three weeks this drug does increase the release of noradrenaline, particularly in the midbrain and brainstem regions, since the steady state concentration of normetanephrine was increased. Mianserin therefore has a different neurochemical profile from that of amitriptyline, but like amitriptyline increases the concentration of brain noradrenaline (though by a different mechanism).

As both amitriptyline and mianserin have behaved identically in the tests described in this paper and in others (van Riezen *et al.,* 1976) the model has withstood the challenge of a novel antidepressant which was not predicted using coventional screening methods.

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Please send reprint requests to A.F.W.

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# EFFECT OF ISOPRENALINE AND PHENYLEPHRINE ON THE ADENOSINE 3′,5′-MONOPHOSPHATE CONTENT AND MECHANICAL ACTIVITY OF COLD-STORED AND FRESH TAENIA CAECUM FROM THE GUINEA-PIG

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1 Cold storage treatment of the guinea-pig taenia caecum had a greater inhibitory effect on the isoprenaline-induced relaxation than that induced by phenylephrine. Prolonged cold storage (12–14 days) almost abolished the effect of isoprenaline but only reduced the phenylephrine effect. The $ED_{50}$ of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) that elicited muscle relaxation was not altered by the prolonged cold storage.

2 After cold storage treatment, tissue cyclic AMP content was decreased; however, isoprenaline still caused a dose-dependent increase in the cyclic AMP level. The threshold dose of isoprenaline for cyclic AMP accumulation was the same in fresh and cold-stored preparations.

3 In the fresh preparation, the onset of the isoprenaline ($10^{-6}$ M)-induced relaxation preceded the increase in tissue cyclic AMP.

4 Isoprenaline, phenylephrine, adrenaline and noradrenaline at doses ($ED_{50}$) sufficient to induce muscle relaxation did not always increase the cyclic AMP level.

5 Similarly, the responses to papaverine and nitroglycerine were not accompanied by an increase in cyclic AMP.

6 The adenylate cyclase and phosphodiesterase (low and high *Km*) activities of taenia caecum were not attenuated by the prolonged cold storage.

7 Propranolol inhibited both the isoprenaline-induced relaxation and cyclic AMP accumulation; however, the $pA_2$ values were significantly different for the two events.

8 Based on these results, both the relaxation and cyclic AMP accumulation caused by isoprenaline are mediated by activation of β-adrenoceptors but are independent phenomena.

## Introduction

Evidence has been presented that in various smooth muscles stimulation of β-adrenoceptors by catecholamines is accompanied by an increase in intracellular cyclic adenosine 3′,5′-monophosphate (cyclic AMP) (Butcher, Ho, Meng & Sutherland, 1965; Bueding, Butcher, Hawkins, Timms & Sutherland, 1966; Robison, Butcher & Sutherland, 1971; Andersson, Lundholm, Mohme-Lundholm & Nilsson, 1972; Bar, 1974). It has also been reported that the relaxation of intestinal smooth muscle induced by β-adrenoceptor stimulation is associated with an increase in cyclic AMP concentration (Andersson & Mohme-Lundholm, 1970; Andersson, 1972; Takayanagi, Uchida, Inatomi, Tomiyama & Takagi, 1972; Inatomi, Takayanagi, Uchida & Takagi, 1974). However, the correlation between cyclic AMP production and intestinal smooth muscle relaxation as effected by β-adrenoceptor agonists has not been fully established and there is still some question as to whether these effects reflect a parallel process or represent a cause and effect.

Our previous data showed that cold treatment of the guinea-pig taenia caecum for 7 days does not interfere with the inhibitory action of the catecholamines but does affect that of adenosine triphosphate and cyclic AMP. This suggested that the underlying mechanisms mediating the catecholamine relaxation may not involve an adenine nucleotide or related compound (Shibata, Hattori & Timmerman, 1970). Moreover, the electrochemical activity following $\alpha$-adrenoceptor stimulation was more resistant to cold storage than was the $\beta$-adrenoceptor response (Fukuda & Shibata, 1972). Therefore, in the experiments described here, the relationship between cyclic AMP production and relaxation was investigated.

## Methods

### *Mechanical activity*

Adult guinea-pigs weighing 500 to 600 g were used in the experiments. The animals were stunned, exsanguinated and the taenia dissected from the caecum. To study the effects of drugs on the mechanical response, taenia strips of approximately 0.5 cm resting length, were suspended vertically in a 50 ml organ bath by silk ligatures, the lower end secured to a holder at the bottom of the chamber and the upper end connected to a Grass strain gauge, force transducer (FT.03) which led to a Grass model 7 polygraph. The bathing medium was Ringer solution of the following composition (mM): NaCl 154, KCl 5.4, $CaCl_2$ 2.4, $NaHCO_3$ 6, glucose 11, bubbled with a gas mixture (95% $O_2$, 5% $CO_2$) and maintained at 37°C and pH 7.4.

A 3 g tension was initially applied to the taenia strip and an equilibration period of 3 h was allowed before the tissue was exposed to any drug. The cumulative dose-response curves were obtained by a stepwise incremental addition of the agonist. Unless specified, the maximum relaxation evoked by phenylephrine in the fresh preparation served as the 100% control to determine the dose-response curves for the different agonists.

In some experiments, the tissues were treated with $\beta$-adrenoceptor blocking agents for 15 min before application of the agonists. The $pA_2$ value was calculated as the negative logarithm of that concentration of the antagonist propranolol, $10^{-6}$ M or $10^{-7}$ M, which requires twice as high a concentration of the agonists to elicit a given response (Rossum, 1963).

For cold storage treatment, the taenia caecum was placed in 250 ml Krebs solution and stored in a refrigerator (set at $2 \pm 0.5$°C) for 1–14 days. After cold treatment, the taenia strip was equilibrated in aerated Ringer at room temperature for 1 h and transferred into the warm medium (37°C) for an additional 2 h before the start of the experimental procedure. $ED_{50}$ values were based on geometric means.

### *Biochemical assay*

Attempts were made to perform all biochemical assays under conditions similar to those in the physiological experiments. The time course of cyclic AMP accumulation and relaxation for the various agonists was carried out simultaneously in different preparations but on tissues from the same animal. For measurements of cyclic AMP, the tissues were exposed to the agonists for 30 s or 1 min, which was sufficient time to cause a maximal relaxation.

### *Cyclic AMP*

Immediately after the incubation or following exposure to the agonists, the taenia strip was pressed between two plates of dry ice. The weighed tissue sample (200–300 mg, 4–5 tissues together) was homogenized in cold 6% trichloroacetic acid and prepared for centrifugation (2000 *g*). The supernatant was extracted with ether (4 times) to remove the trichloroacetic acid before being subjected to the lyophilization procedure. The residue from the process was then dissolved in buffer, and a suitable aliquot was taken for cyclic AMP determination by the protein binding method. The binding protein was prepared from rat brain (Miyamoto, Kuo & Greengard, 1969) and the assay was carried out essentially as described by Gilman (1970).

### *Adenylate cyclase and phosphodiesterase*

The taenia caecum was first homogenized in ice-cold Tris-maleate buffer (pH 7.4) containing 2 mM 1,2, bis, 2 aminoethoxyethane-NNN'N'-tetra-acetic acid (EGTA) and then filtered through a gauze. A portion of the filtrate was taken for the phosphodiesterase (PDE) assay; the remainder was centrifuged at 4°C for 10 min at 2500 rev/min and the precipitate was resuspended in 5 mM Tris-maleate-2 mM EGTA buffer and used for the determination of adenylate cyclase activity.

The adenylate cyclase assay was a modified method of Kebabian, Petzold & Greengard (1972). The reaction mixture (0.6 ml) consisted of 80 mM Tris-maleate (pH 7.4), 2 mM $MgSO_4$, 10 mM theophylline, 0.2 mM EGTA, 10 mM creatine phosphate, 6 i.u. of creatine phosphokinase, 0.5 mM ATP and the enzyme preparation (ca. 2 mg as protein). The reaction was started by the addition of ATP in the presence of 10 mM NaF. After incubation at 30°C for 2.5 min, the enzyme mixture was immersed for 2 min in a boiling water bath to stop the reaction and then centrifuged. The resultant supernatant was taken and the amount of cyclic AMP produced was determined as described above. The enzyme activity is expressed as picomoles of cyclic AMP formed during 2.5 min/mg of protein. Protein content was determined by a Biuret method described by Gornall, Bardawill & David (1949).

PDE assay was carried out in Tris-HCl buffer (40 mM, pH 7.5), containing 1 mM $MgCl_2$. The high *Km*-PDE activity was determined as described by

Butcher & Sutherland (1962) with 2 mM cyclic AMP substrate. Inorganic phosphate determination was by the method of Post & Sen (1967). The low *Km*-PDE was assayed according to the method of Thompson & Appleman (1971) with [$^3$H]-cyclic AMP at a concentration of 1.5 x $10^{-7}$ M. The high *Km*-PDE activity was expressed as nanomoles of cyclic AMP hydrolyzed during 10 min/mg of protein and the low *Km*-PDE activity was expressed as picomoles of cyclic AMP hydrolyzed during 10 min/mg of protein.

The following agents were used: (±)-isoprenaline sulphate, (−)-phenylephrine hydrochloride, (−)-noradrenaline bitartrate, (−)-adrenaline bitartrate, papaverine hydrochloride, nitroglycerine (2% ethanol solution) and adenosine 3′,5′-cyclic monophosphoric acid (cyclic AMP) (Sigma). Propranolol hydrochloride (Ayerst Lab) was used as a $\beta$-adrenoreceptor blocking agent.

## Results

### *Relaxant effect of isoprenaline, phenylephrine and cyclic AMP*

Figure 1 illustrates the dose-related relaxant effect of isoprenaline and phenylephrine on the guinea-pig taenia strips before and after cold storage treatment. Cold storage of the taenia strip for 9 days caused the dose-response curve of isoprenaline to shift to the right along with a slight attenuation of the maximum relaxant effect, whereas such treatment had little effect on the dose-response curve of phenylephrine and the maximum efficacy of phenylephrine was not appreciably affected. Even after prolonged cold storage (12–14 days), the maximum relaxant effect evoked by phenylephrine was diminished by only 25%, whereas the response to isoprenaline was barely detectable.

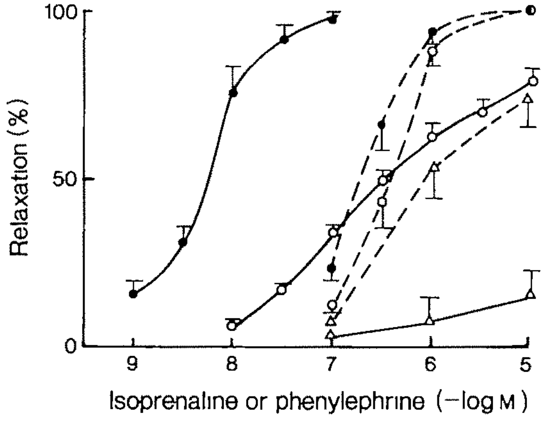

**Figure 1** Relaxant effect of isoprenaline and phenylephrine on the fresh and cold-stored guinea-pig taenia caecum. Fresh preparations: (●——●) isoprenaline; (●----●) phenylephrine. Cold-stored strips: (○——○) isoprenaline 9 days, (△——△) isoprenaline 12–14 days; (○----○) phenylephrine 9 days; (△---△) phenylephrine 12–14 days. Each plot is the mean of 14 strips. Vertical lines show s.e. mean Different animals were used in each of the experiments.

In Table 1 are shown the $ED_{50}$ for isoprenaline, phenylephrine and cyclic AMP in the fresh and cold-stored taenia. The $ED_{50}$ for isoprenaline in the 9 day cold-stored taenia strips was approximately 100 times that in the fresh preparations. In the 12–14 day-treated strips, the relaxant effect of isoprenaline was so severely depressed that the $ED_{50}$ was not measurable. On the other hand, the reactivity of the taenia strip to phenylephrine after 9 days of cold storage was no different from that of the fresh preparation. Moreover the $ED_{50}$ for phenylephrine in the 12–14 day-treated taenia was only increased twofold over the untreated control. In contrast, the $ED_{50}$ for cyclic AMP was not significantly altered by the cold storage treatment, although the maximal efficacy of cyclic AMP progressively decreased to approximately 52% ($n=7$).

### *Effect of catecholamines on cyclic AMP production*

In Figure 2 are presented the net (a) and percentage (b) increases of tissue cyclic AMP following the

**Table 1** Relaxant effect ($ED_{50}$) of isoprenaline, phenylephrine and cyclic AMP on fresh and cold stored guinea-pig taenia caecum

| | *Fresh* | *Cold stored* | |
|---|---|---|---|
| | | *9 days* | 12–14 days |
| Isoprenaline (M) | $5.5 \pm 1.0 \times 10^{-9}$ | $6.0 \pm 1.2 \times 10^{-7*}$ | ——** |
| Phenylephrine (M) | $3.0 \pm 0.8 \times 10^{-7}$ | $4.0 \pm 0.6 \times 10^{-7}$ | $6.2 \pm 0.6 \times 10^{-7*}$ |
| Cyclic AMP (M) | $4.9 \pm 1.1 \times 10^{-4}$ | $5.1 \pm 0.6 \times 10^{-4}$ | $6.7 \pm 0.8 \times 10^{-4}$ |

*Significantly different from value of fresh tissue (Student *t*-test, $P<0.05$)
**Unmeasurable response.
Each value is mean ± s.e. mean of 7 experiments.

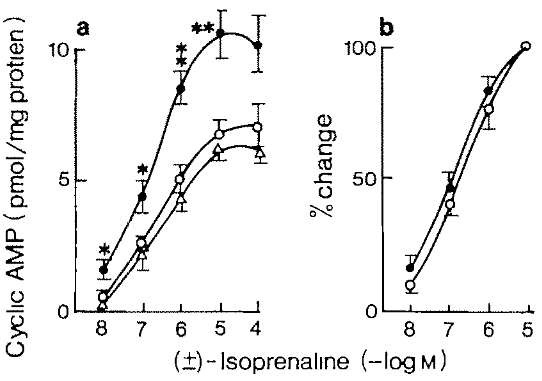

**Figure 2** Effect of isoprenaline on the cyclic AMP content of fresh and cold-stored guinea-pig taenia caecum. (a) Net increase in cyclic AMP content; the cyclic AMP content before exposure to isoprenaline was subtracted from each individual value (see text). (b) Percentage change of net increase in cyclic AMP level. (●) Fresh strips; (○) cold stored strips (9 days); (△) cold stored strips (12–14 days). Each value is the mean of 11 experiments Vertical lines show s.e. mean.

$^{*}P<0.05$ (Student's $t$-test); $^{**}P<0.01$ between fresh and cold-stored strips.

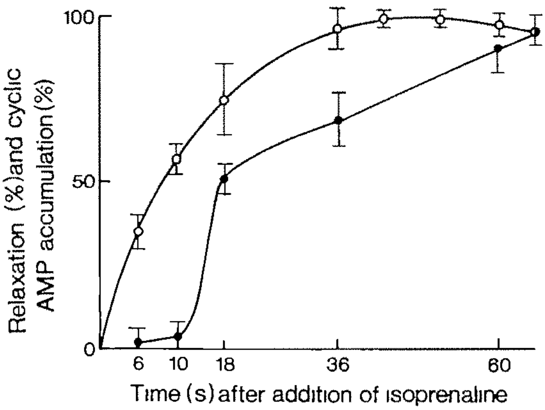

**Figure 3** Time course of isoprenaline ($2\times10^{-7}$ M) induced relaxation and cyclic AMP production (pmol/mg protein) in the guinea-pig taenia caecum. (○) Relaxation; (●) cyclic AMP. Each value is the mean of 7 experiments Vertical lines show s.e. mean Cyclic AMP content of control tissue before exposure to isoprenaline was $9.48\pm0.68$ pmol/mg protein.

application of isoprenaline ($10^{-8}$ M–$10^{-4}$ M) in the fresh and cold-stored taenia strips. Both fresh and cold-stored preparations showed a dose-dependent increase in cyclic AMP content in response to isoprenaline. Although the tissue cyclic AMP content in fresh preparations was always greater than that in the cold-treated taenia in the whole range of isoprenaline concentration examined, the drug effect was not different in the cold-stored preparations. Even at the maximally effective concentrations of isoprenaline the cyclic AMP level was the same in the 9 day and 12–14 day cold-treated taenia strips. A measurable increase in cyclic AMP was detected at isoprenaline $10^{-8}$ M and the maximum effect occurred at a concentration of $10^{-5}$ M in both fresh and cold-treated strips. Other catecholamines such as adrenaline, noradrenaline and phenylephrine at $ED_{50}$ for the relaxation failed to increase significantly the cyclic AMP content of the taenia. Even high concentrations of phenylephrine ($10^{-5}$ M) did not increase the cyclic AMP content. The dose-response curves for isoprenaline in the fresh and cold-treated taenia became almost identical when the drug effect was expressed as a percentage increase in cyclic AMP (Figure 2b). The $ED_{50}$ was $2.6\pm0.6\times10^{-7}$ M ($n=11$) in the fresh taenia and increased slightly to $3.1\pm0.6\times10^{-7}$M ($n=11$) with cold storage. Apparently cyclic AMP biosynthesis was not adversely affected by cold storage, as the adenylate cyclase activity and the low and high *Km*-PDE values in the cold-stored strips were not significantly different from those of the fresh preparations. Adenylate cyclase activity in the fresh tissue was $219\pm18$ pmol cyclic AMP formed and in the cold-treated strip $225\pm36$. The PDE values for low and high *Km* were $252\pm15$ pmol/mg$^{-1}$ protein 10 min$^{-1}$ and $129\pm15$ nmol/mg$^{-1}$ protein 10 min$^{-1}$ for the fresh and $278\pm32$ and $121\pm7$ for the cold-treated taenia, respectively. The cyclic AMP content meanwhile progressively declined from $9.73\pm0.57$ pmol/mg protein ($n=18$) in the fresh preparations to $6.22\pm0.47$ ($n=18$) in the 9 day and $6.08\pm0.32$ in the 12–14 day cold-stored preparations.

### *Time course of relaxation and cyclic AMP accumulation*

Figure 3 illustrates the time course of relaxation and the changes in the tissue cyclic AMP following the administration of isoprenaline ($2\times10^{-7}$ M). In all cases, the relaxation occurred rapidly and preceded the increase in cyclic AMP. Within 6 to 10 s after the isoprenaline application, the relaxation was already approximately 35% and 57% of maximum, while the cyclic AMP content was not yet significantly increased. The inhibitory response reached a maximum after about 36 s and remained at this level for approximately 2 min when the cyclic AMP content was still increasing.

### *Antagonistic effect of propranolol on the isoprenaline-induced relaxation and cyclic AMP accumulation*

Pretreatment with propranolol ($10^{-7}$ and $10^{-6}$ M) for 15 min competitively inhibited the relaxant effect of

**Table 2** The antagonistic effect of propranolol on the isoprenaline-induced relaxation of the taenia caecum

| | *Relaxation (%)* | | |
|---|---|---|---|
| | | *Treatment with propranolol* | |
| *Isoprenaline* (M) | *Control* | *1 × $10^{-7}$*M | *1 × $10^{-6}$*M |
| $10^{-9}$ | 5.6 ± 1.1 | 3.1 ± 1.6 | 2.2 ± 0.7 |
| 3 × $10^{-9}$ | 16.1 ± 2.2 | 6.3 ± 2.0 | 5.3 ± 1.6 |
| $10^{-8}$ | 34.0 ± 4.8 | 10.8 ± 2.4 | 8.8 ± 1.6 |
| 3 × $10^{-8}$ | 53.2 ± 5.6 | 19.0 ± 3.1 | 13.6 ± 1.6 |
| $10^{-7}$ | 78.6 ± 2.7 | 34.2 ± 4.4 | 20.0 ± 2.2 |
| 3 × $10^{-7}$ | 91.9 ± 2.7 | 56.7 ± 6.2 | 32.6 ± 2.9 |
| $10^{-6}$ | 100 | 79.9 ± 4.2 | 58.4 ± 4.2 |
| 3 × $10^{-6}$ | | 93.7 ± 1.7 | 72.4 ± 3.2 |
| $10^{-5}$ | | 100 | 90.5 ± 2.5 |
| 3 × $10^{-5}$ | | | 100 |

The maximum relaxation in response to isoprenaline served as 100% control

Each value expresses mean ± s.e mean of 16 and 12 experiments in control and propranolol treatment, respectively.

**Table 3** The antagonistic effect of propranolol on the isoprenaline-enhanced cyclic AMP content of the taenia caecum

| | *Cyclic AMP (pmol/mg of protein* | |
|---|---|---|
| *Isoprenaline* (M) | *No treatment* | *Propranolol* ($10^{-7}$M) |
| Control A | 5.69 ± 0.53 | ——— |
| Control B | ——— | 5.08 ± 0.34 |
| $10^{-8}$ | 6.70 ± 0.52 | 5.68 ± 0.34 |
| $10^{-7}$ | 6.36 ± 0.41 | 5.84 ± 0.57 |
| $10^{-6}$ | 10.50 ± 0.73 | 6.79 ± 0.50 |
| $10^{-5}$ | 10.81 ± 0.90 | 7.28 ± 0.91 |
| $10^{-4}$ | 10.93 ± 0.11 | 10.50 ± 1.1 |

Control A was not exposed to any agent; Control B was exposed to propranolol only

Tissues were exposed to isoprenaline 15 min after treatment with propranolol. The tissues were exposed to isoprenaline for 1 minute. The full scale relaxation was observed within 1 min after application of isoprenaline. For each experiment, the taeniae from five different animals were used. Medium contained 2 mM theophylline. Each value of cyclic AMP is the mean ± s.e. mean of 7 experiments

various concentrations ($10^{-9}$ M to 3 × $10^{-5}$ M) of isoprenaline in the fresh taenia (Table 2). Similar treatment also inhibited the increase in cyclic AMP accumulation (Table 3).

The corresponding $pA_2$ values for the propranolol-isoprenaline interaction were 7.92 ± 0.10 for relaxation and 8.57 ± 0.14 for cyclic AMP accumulation.

### *Effect of papaverine and nitroglycerine on mechanical activity and cyclic AMP content*

Papaverine (5 × $10^{-7}$ M – 5 × $10^{-5}$ M) and nitroglycerine (3 × $10^{-8}$ M – 3 × $10^{-6}$ M) also caused a similar dose-dependent relaxation of the taenia caecum; however, these agents did not increase the tissue cyclic AMP content (Table 4). For both drugs, the cyclic AMP level even at concentrations that elicited maximal relaxation was not significantly greater than the control.

## Discussion

Early studies on the effects of catecholamines in smooth muscle demonstrated that adrenaline at doses sufficient to cause muscle relaxation induced a slight but significant increase in tissue cyclic AMP content (Butcher *et al.*, 1965; Bueding *et al.*, 1966). More

**Table 4** Effect of papaverine and nitroglycerine on the mechanical activity and cyclic AMP level of the guinea-pig taenia caecum

| *Treatment* (M) | *Relaxation* (%) | *Cyclic AMP* (pmol/mg protein) |
|---|---|---|
| Control | 0 | 10.9 ± 1.0 |
| Papaverine | | |
| $5 \times 10^{-7}$ | 10 | 10.5 ± 0.6 |
| $5 \times 10^{-6}$ | 50 | 11.0 ± 1 2 |
| $5 \times 10^{-5}$ | 100 | 10.6 ± 0.9 |
| $5 \times 10^{-5}$ (3 min) | 100 | 12 1 ± 1 3 |
| Nitroglycerine | | |
| $3 \times 10^{-8}$ | 2 | 11.1 ± 0.4 |
| $3 \times 10^{-7}$ | 45 | 10.4 ± 1.3 |
| $3 \times 10^{-6}$ | 100 | 11.0 ± 1.6 |

Controls were exposed to neither agent. All experiments were carried out in the presence of theophylline (2 mM). The tissues were exposed to agents for 1 min or as specified in parentheses. The full scale relaxation was observed within 1 min after application of papaverine or nitroglycerine. Each value of cyclic AMP is the mean s.e. mean of 7–10 experiments. For each experiment, the taeniae from five different animals were used.

recently, several investigators have reported that the relaxation induced by a $\beta$-adrenoceptor stimulation is accompanied by an increase in tissue cyclic AMP in rabbit colon and guinea-pig taenia caecum (Andersson & Mohme-Lundholm, 1969; 1970; Andersson, 1972; Takayanagi *et al.*, 1972; Inatomi *et al.*, 1974). These results suggest that cyclic AMP may be the intracellular mediator of the intestinal smooth muscle relaxation produced by $\beta$-adrenoceptor activation. However, the relationship between cyclic AMP accumulation and intestinal muscular relaxation has not been carefully examined and the question still arises as to whether they are independent effects or causally interrelated.

As described previously (Fukuda & Shibata, 1972; Hattori, Kurahashi, Mori & Shibata, 1972) cold-storage treatment of the taenia caecum caused a differential effect on the isoprenaline- and phenylephrine-induced relaxation. The cold treatment reduced the efficacy as well as potency of isoprenaline, whereas it had much less effect on the response to phenylephrine, suggesting that the muscle relaxation through $\alpha$- and $\beta$-adrenoceptors involves separate mechanisms. Cold storage decreased the basal cyclic AMP content of the taenia caecum; however, even in the 12–14 day cold-stored tissues in which muscle relaxation was barely measurable, isoprenaline still caused a prominent dose-related increase in cyclic AMP. In fact the $ED_{50}$ of isoprenaline for tissue cyclic AMP increase was the same for the fresh and cold-stored preparations (Figure 2). These results would suggest a dissociation of the isoprenaline-induced events: muscle relaxation and cycle AMP accumulation,

In the fresh taenia strips the isoprenaline dose-relaxation curve was not correlated with that for cyclic AMP accumulation. At a concentration corresponding to the $ED_{50}$ for muscle relaxation, isoprenaline did not cause an observable increase in cyclic AMP and the concentration for maximum relaxation was approximately 100 times less than that required for maximum cyclic AMP increase. Similarly, other catecholamines such as adrenaline, noradrenaline and phenylephrine at muscle relaxing concentrations failed to increase the cyclic AMP level. Therefore, it would appear that even in the fresh tissue relaxation and cyclic AMP accumulation can be separate events.

In the rabbit colon an increase in cyclic AMP preceded the relaxation induced by isoprenaline (Andersson & Mohme-Lundholm, 1969; Andersson, 1972). Conversely, in the present experiments, the relaxation started before any increase in cyclic AMP. The onset of the relaxant effect was not in phase with cyclic AMP production.

The antagonism of the isoprenaline-induced relaxation and cyclic AMP accumulation by propranolol would indicate that both phenomena are mediated through activation of $\beta$-receptors. However, the $pA_2$ value for cyclic AMP accumulation was much greater than for the relaxation effect. Presumably, the $\beta$-adrenoceptors that stimulate the relaxing mechanism have different properties from the receptors that activate cyclic AMP production. This would explain the lack of inhibitory effect of exogenously administered dibutyryl cyclic AMP on intestinal smooth muscle (Levine, 1968; Andersson & Mohme-Lundholm, 1970; Fukuda & Shibata, 1972).

Takayanagi *et al.* (1972) and Inatomi *et al.* (1974) showed that papaverine increases the intracellular cyclic AMP level in guinea-pig taenia caecum and Andersson (1973) reported that the relaxation of the rabbit colon by papaverine and nitroglycerine is preceded by an increase in the nucleotide. However, in the present investigation the relaxation by papaverine and nitroglycerine occurred without any accompanying change in tissue cyclic AMP, adenylate cyclase or PDE activity. These contrasting results may be explained by the different drug concentrations employed. In these experiments the concentrations of papaverine and nitroglycerine were approximately 5 to 200 times less than employed by Takayanagi *et al.* (1972), Inatomi *et al.* (1974) and Andersson (1973). In this regard, Polacek, Bolan & Daniel (1971) observed little or no elevation of tissue cyclic AMP level in the rat uterus with low but relaxation-producing concentrations of papaverine. Therefore, the present data also provide evidence that the relaxation induced by papaverine and nitroglycerine is not mediated by an increase in tissue cyclic AMP.

The cold-storage treatment did not alter the adenylate cyclase or phosphodiesterase (low *Km* and high *Km*) activities in the taenia. Kuchii, Miyahara & Shibata (1973) previously demonstrated that cold storage had little effect on the biotransformation of adenosine to adenosine triphosphate in the taenia caecum. Therefore, the low level of cyclic AMP in the cold-stored tissue compared to the fresh preparation probably stems from a decrease in endogenous ATP.

The present study was concerned with measurement of total tissue cyclic AMP and does not eliminate the possibility that cyclic AMP changes in subcellular compartments may be related to the relaxant effect.

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# AGONIST AND ANTAGONIST PROPERTIES OF BUPRENORPHINE, A NEW ANTINOCICEPTIVE AGENT

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**1** Buprenorphine is a highly lipophilic derivative of oripavine. In rodent antinociceptive assays (writhing, tail pressure), buprenorphine had an action which was rapid in onset and of long duration; it was 25–40 times more potent than morphine after parenteral injection and 7–10 times more potent after oral administration.

**2** The log dose-response relationship for buprenorphine was curvilinear in mouse and rat tail flick tests with the antinociceptive effect decreasing at higher, non-toxic doses.

**3** Tolerance developed to the antinociceptive activity of buprenorphine in mice.

**4** No signs of abstinence were observed on naloxone challenge or after abrupt withdrawal in monkeys receiving buprenorphine chronically for one month.

**5** Buprenorphine antagonized the antinociceptive actions of morphine in mouse and rat tail flick tests but was an ineffective antagonist in the rat tail pressure test.

**6** Buprenorphine precipitated signs of abstinence in morphine-dependent mice and monkeys but not in morphine-dependent rats.

**7** Buprenorphine produced Straub tails in mice. This effect was not antagonized when the animals were pretreated with naloxone. However, in the rat tail pressure test high doses of diprenorphine antagonized established antinociceptive effects of buprenorphine.

**8** It is concluded that buprenorphine represents a definite advance in the search for a narcotic antagonist analgesic of low physical dependence potential.

## Introduction

In the search for alternative analgesics to morphine, interest has continued to focus on those compounds that possess both antinociceptive and narcotic antagonist properties. Much research has been sustained by the conviction that a certain balance of these two activities will produce an efficacious analgesic that lacks both the physical dependence potential of morphine and the psychotomimetic profile of cyclazocine. This belief has been reinforced during the pharmacological evaluation of buprenorphine, N-cyclopropylmethyl-7$\alpha$-(1-*S*-hydroxy,1,2,2-trimethylpropyl)-6,14-*endo*ethano-6,7,8,14-tetrahydronororipavine (RX 6029-M), a new narcotic antagonist with potent, long-lasting antinociceptive actions that are not associated with an ability to induce primary physical dependence in monkeys. The similarity in chemical structure between buprenorphine and the potent narcotic antagonist, diprenorphine (M5050), is shown in Figure 1.

A preliminary communication on the agonist and antagonist effects of buprenorphine in animals was given at the First International Conference on Narcotic Antagonists, Warrenton, U.S.A. (November, 1972). In the present paper, the antinociceptive and narcotic antagonist properties of buprenorphine are fully described and the physical dependence liability of this new analgesic is assessed.

### *Animals*

The experiments were carried out on albino mice (MFI/Ola, 18–24 g), male Sprague Dawley albino rats in the weight ranges indicated below, monkeys (*Erythrocebus patas*, 4–10 kg) and baboons (*Papio anubis*, 4–7 kg).

### *Compounds*

The following compounds were dissolved or diluted in 0.9% w/v NaCl solution (saline) and the doses calculated in terms of the free base: buprenorphine hydrochloride (mol. wt. of base is 467.6) and

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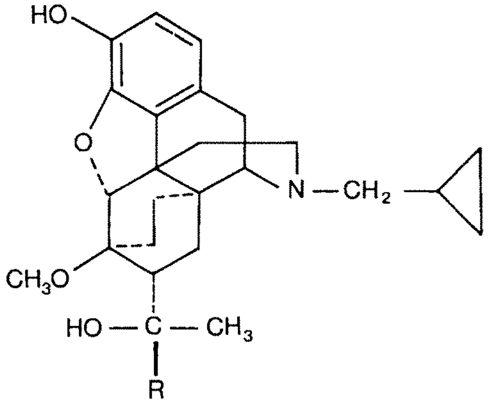

Buprenorphine: R = *t* butyl
Diprenorphine: R = methyl

**Figure 1** Structural diagrams of buprenorphine and diprenorphine.

diprenorphine hydrochloride (both Reckitt & Colman), morphine sulphate, B.P. (Macfarlan Smith), nalorphine hydrobromide, B.P. (Burroughs Wellcome), naloxone hydrochloride (Endo), pentazocine lactate (Sterling-Winthrop) and phenazocine hydrobromide (Smith & Nephew).

The free base of cyclazocine (Sterling-Winthrop) was dissolved in a minimal amount of 0.1 N HCl, the pH adjusted to 5.0 with $NaHCO_3$ solution and made up to volume with saline. Phenylquinone (2-phenyl-1,4-benzoquinone, Eastman) was dissolved in 5% w/v ethanol in distilled water.

### *Antinociceptive tests*

In the writhing test, each dose of compound was administered to different groups of 10 female mice at various times before challenge with phenylquinone (2 mg/kg, i.p.). Five min later, the number of abdominal writhing movements made by each mouse was counted for 10 minutes. The dose of compound required to reduce the incidence of writhing by 50% was calculated by comparison with saline-injected controls (Hendershot & Forsaith, 1959). The development of tolerance to analgesics was monitored by this procedure. Thus, 2 groups of 240 mice were injected subcutaneously twice daily, at 09 h 00 min and 16 h 00 min, for 7 consecutive days with either buprenorphine (0.90 mg/kg i.e. 100 × the antinociceptive $ED_{50}$ dose at 2 h, the time of peak effect) or morphine (56 mg/kg i.e. 100 × the $ED_{50}$ dose at 0.5 h, the time of peak effect). Regression lines for antinociceptive effect were obtained daily at 10 h 00 min, with 24–30 mice from each group.

In the tail flick test, the noxious stimulus was hot water maintained at either 45, 55 or 65 °C. Only mice and rats (120–150 g) withdrawing their tails from the water within 2 s were used. Immediately after selection, test compounds were administered intraperitoneally and 30 min later reaction times were again noted. On the basis of control data, those mice or rats failing to withdraw their tails within 7.5 s (45 °C), 5.0 s (55 °C) or 3.0 s (65 °C), respectively, were termed 'non-responders'. Antagonism of the antinociceptive effect of morphine was also studied with this procedure. Either saline or test compound was given intraperitoneally to groups of 10 mice or rats at various times before subcutaneous injection of the antinociceptive $ED_{90}$ dose of morphine. Reaction times (to water maintained at 55 °C) were noted 30 min later. The $AD_{50}$ and $AD_{80}$ values are defined as the doses of antagonist required to reduce by 50% and 80%, respectively, the number of animals no longer responding to a noxious stimulus following treatment with the $ED_{90}$ dose of morphine.

Groups of 10 rats (40–60 g) were used in the version of the tail pressure test described by Boura & Fitzgerald (1966). For antagonism studies, rats received the $ED_{90}$ dose of morphine (s.c.) followed immediately by either saline or antagonist (s.c.). The analgesic test took place 30 min later.

### *Direct dependence test*

Six patas monkeys were injected subcutaneously at 09 h 00 min, 16 h 00 min and 22 h 30 min for 30–32 consecutive days with increasing doses of buprenorphine (1.5–3.0 mg/kg in 2 monkeys and 3.0–12.5 mg/kg in 4 monkeys). The development of physical dependence was monitored by challenging each primate with naloxone (2 mg/kg, s.c.) on days 14 and 28 and looking for the signs of abstinence listed by Deneau & Seevers (1964). Saline was injected during the week following abrupt withdrawal of buprenorphine and the animals were frequently observed through a one-way mirror for signs of withdrawal.

### *Tests with morphine-dependent animals*

The method described by Cowan (1976) was used to obtain an extended dose-response curve for buprenorphine as a morphine antagonist in the mouse jumping test.

Groups of 8 rats (140–170 g) were implanted in the dorsal subcutaneous tissue with a morphine pellet (75 mg morphine base, 2 mg polyvinylpyrrolidone and 1 mg magnesium stearate) on day 1 and again 48 h later. A further 24 h later each rat was weighed then challenged subcutaneously with antagonist or saline. Animals were placed in individual open plastic containers (43 cm long, 30 cm wide and 17 cm high) and observed over 10 min for signs of abstinence (Bläsig, Herz, Reinhold & Zieglgänsberger, 1973).

Food and water were removed and the weight of each rat was recorded 3 and 24 h later.

Five non-withdrawn, morphine-dependent patas monkeys that had received daily injections of the narcotic at 09 h 00 min, 16 h 00 min (6 mg/kg, s.c.) and 22 h 30 min (12 mg/kg) for 15 months were injected with buprenorphine (0.01–10 mg/kg, s.c.) at 11 h 00 minutes. The resulting abstinence syndromes were classified according to the protocol described by Deneau & Seevers (1964).

### *Straub tail and catalepsy tests*

Thirty min after subcutaneous injection of buprenorphine, mice ($n = 10$) were individually observed for 30 s and those with tails elevated >45° to the horizontal were scored as showing a positive Straub effect. In antagonism studies, the antagonist was given subcutaneously to groups of 10 mice, 15 min before the various doses of buprenorphine.

The cataleptic effects of buprenorphine and morphine were estimated 0.5, 1.5 and 2.5 h after subcutaneous administration to groups of 20 rats (120–150 g). The number of animals leaving both hind legs over a horizontal metal rod (4 cm above bench level) for longer than 45 s was recorded.

### *Statistical evaluation*

Quantal data were subjected to logit analyses using Bliss 17, a computer programme written by Professor D.J. Finney (see Finney, 1971). Figures in parentheses in the Tables refer to the 95% confidence limits of the AD$_{50}$ or ED$_{50}$.

## Results

The potencies of buprenorphine and reference analgesics in the phenylquinone test are presented in Table 1. The slope values of the narcotic antagonist analgesics did not differ significantly from that of morphine at 30 minutes. A fast onset of action was noted for buprenorphine; the subcutaneous ED$_{50}$ value obtained at 5 min (0.019 mg/kg, 0.008–0.043, 95% confidence limits) was close to the optimal value obtained at 2 h (0.008 mg/kg, 0.005–0.013). In contrast to findings from other procedures (see below),

**Table 1** The potencies of analgesics against abdominal constriction responses induced by phenylquinone in mice

| *Compound* | *Median effective antinociceptive dose* | | | | |
|---|---|---|---|---|---|
| | *ED$_{50}$ (mg/kg, s.c.)* | | | *ED$_{50}$ (mg/kg, orally)* | |
| | *0.5 h* | *Slope* | *2.0 h* | *1.0 h* | *2.0 h* |
| Buprenorphine | 0.014<br>(0.007–0.027) | 1 3 | 0.008<br>(0.005–0.013) | 0.39<br>(0.25–0.63) | 0 24<br>(0.14–0.39) |
| Cyclazocine | 0.22<br>(0.11–0.42) | 1.3 | 4.6<br>(2.1–10) | 5.2<br>(2.9–9.4) | 27<br>(19–39) |
| Morphine | 0.36<br>(0.22–0.60) | 1.8 | 2.4<br>(1.4–4 0) | 2 6<br>(1.4–4.9) | 9.6<br>(6.3–14) |
| Pentazocine | 4.3<br>(2.6–7.1) | 1.9 | >60 | 59<br>(46–76) | >60 |

Different groups of mice were used at each time interval. The slopes of the antinociceptive regression lines, computed from the readings at 0.5 h, are expressed as logits/log$_e$ dose Figures in parentheses refer to 95% confidence limits.

**Table 2** The antinociceptive potencies of analgesics in the mouse and rat tail flick tests

| *Compound* | *Mouse tail flick test ED$_{50}$ (mg/kg, i.p.)* | *Rat tail flick test ED$_{50}$ (mg/kg, i.p.)* |
|---|---|---|
| Buprenorphine | 2.4(0.25–22) | 1 6(0 71–3 6) |
| Morphine | 3.8(2.0–7 4) | 9 5(4.8–19) |
| Pentazocine | >30 | >30 |

The noxious stimulus was water maintained at 55°C. In both tests, the log dose-response curve for buprenorphine was curvilinear. The so-called ED$_{50}$ values for buprenorphine were calculated from that part of the curve where the antinociceptive effect increased with dose. Figures in parentheses refer to 95% confidence limits.

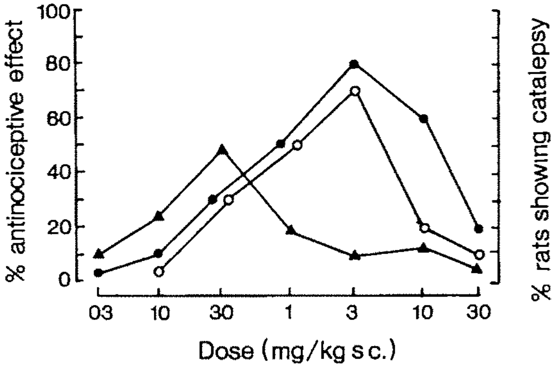

**Figure 2** Dose-response curves obtained with buprenorphine (I) in the rat tail flick test using water at 45°C (●) or 55°C (○) as the noxious stimulus and (II) in a catalepsy test (▲) where those rats that left both hind legs over a horizontal rod >45 s were scored as showing a positive effect. Ten rats were used for each point in the analgesic test, 20 rats were used per point in the catalepsy test. All readings were taken 30 min after injection.

buprenorphine maintained a 100% effect after high doses (0.10–100 mg/kg) in the phenylquinone test.

Buprenorphine was marginally more potent than morphine in the mouse and rat tail flick tests when water at 55°C was used as the noxious stimulus; in both cases, the maximal effect of pentazocine was only 40% and so $ED_{50}$ values could not be calculated (Table 2). The efficacy (maximal effect) of buprenorphine was increased when the noxious stimulus was set at 45°C (Figure 2). Under these conditions pentazocine was weakly active, a 50% response being obtained. Buprenorphine was less efficacious than morphine in mice and rats; the maximal effect of the new compound, with water at 65°C, was only a 50% suppression of the flick reflex.

The log dose-response lines for morphine at 45, 55 and 65°C were of the typical sigmoid shape. In contrast, those for buprenorphine at each of these temperatures, and in both mice and rats, were bell-shaped with the antinociceptive effect decreasing at higher, non-toxic doses. A similar bell-shaped curve was obtained with pentazocine at 45°C. It is of interest that the dose of buprenorphine (3 mg/kg) producing the maximal antinociceptive effect in rats was 10 times higher than the dose causing the maximal cataleptic effect in this species (Figure 2). Thus, in view of the bell-shaped dose-response curve for catalepsy, only 10% of the animals were classified as being cataleptic at the peak antinociceptive dose level.

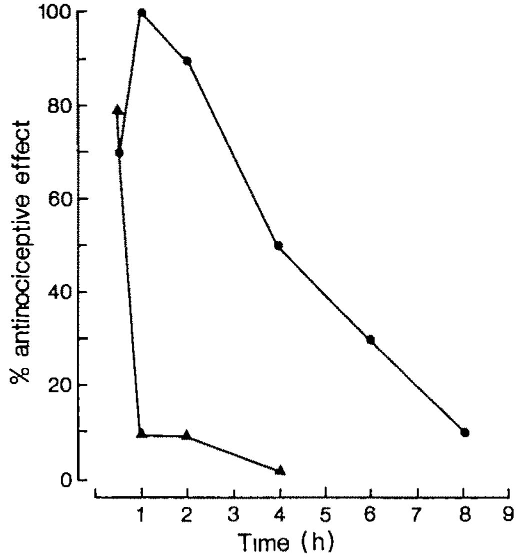

**Figure 3** Comparison of the lengths of action of buprenorphine (●) and morphine (▲) as antinociceptive agents in the rat tail pressure test. The $ED_{80}$ agonist doses (previously estimated to be 0.046 and 1.0 mg/kg, s.c. at 0.5 h for buprenorphine and morphine, respectively) were injected into different groups of 10 rats at zero time Each group was used at only one time interval. The number of rats in each group that did not respond to the noxious stimulus at specified times (abscissa scale) is plotted on the ordinate scale.

Buprenorphine was more potent as an antinociceptive agent than morphine and, especially, pentazocine in the rat tail pressure test (Table 3). A rapid onset of action occurred since the $ED_{50}$ value at 5 min was 0.053 mg/kg (0.016–0.18). There was no evidence of a bell-shaped dose-response curve in this procedure; a consistent 100% antinociceptive effect

**Table 3** The antinociceptive potencies of analgesics in the rat tail pressure test

| *Compound* | *$ED_{50}$ (mg/kg, i.p.)* | *$ED_{80}$ (mg/kg, orally)* |
|---|---|---|
| Buprenorphine | 0.016(0.011–0.024) | 0.35(0 16–0 77) |
| Morphine | 0 66(0.26–1.6) | 3.6(1.6–8.1) |
| Pentazocine | 8.8(3.5–22) | 35(16–76) |

Antinociceptive effects were recorded 0.5 h after intraperitoneal injection and 1.0 h after oral administration. Figures in parentheses refer to 95% confidence limits.

was obtained at 30 min with animals receiving higher doses of buprenorphine (0.10–100 mg/kg). The duration of action of buprenorphine (based on the time taken for the $ED_{80}$ dose to be effective in only 20% of the rats) was 9 or 13 times longer than that of morphine, the choice depending on which of two possible times is taken as zero for buprenorphine (Figure 3).

Two morphine-like properties of buprenorphine were demonstrated in the phenylquinone-induced writhing test. Tolerance developed to the antinociceptive effect of buprenorphine, the onset being slower than with morphine. Also, cross tolerance was shown to exist between the two analgesics; this was more pronounced in mice pretreated with morphine (Table 4). Despite these findings, multiple injections of buprenorphine did not induce physical dependence in monkeys as judged by the absence of signs of abstinence on naloxone challenge and also after abrupt withdrawal of buprenorphine.

The morphine antagonist activity of buprenorphine was most clearly demonstrated using the mouse tail flick test. Buprenorphine was as potent as naloxone at the time of peak effect (Table 5) and had a duration of action approximately 10 times that of naloxone (Table 6). The maximum antagonistic effect obtained with buprenorphine, however, was only 80% and this was maintained over the dose-range 0.10–100 mg/kg.

**Table 4** Development of tolerance to the antinociceptive actions of buprenorphine and morphine and possible cross tolerance between these compounds in the mouse phenylquinone-writhing test

| *No. of injections of analgesic* | *Antinociceptive $ED_{50}$ (mg/kg, s.c.)* | |
|---|---|---|
| *(a) Tolerance* | *Buprenorphine* | *Morphine* |
| Saline control | 0.013(0.005–0 036) | 0 60(0 40–0 90) |
| 2 | 0 013(0 005–0.034) | 1 1(0 68–1 7) |
| 4 | 0.028(0.016–0 049) | 3.3(1.7–6.7) |
| 6 | 0 083(0 052–0 13) | 6 9(4.3–11) |
| 8 | 0 13(0.078–0.21) | 8.2(6.0–11) |
| 14 | 5.6(3.2–9.8) | 7.7(3.7–16) |
| *(b) Cross tolerance* | | |
| 14 (morphine) | 0.39(0.21–0.74) | |
| 14 (buprenorphine) | | 6.0(4.1–8.7) |

Each analgesic was injected at 09 h 00 min and 16 h 00 min daily for 7 days The dose level was 100 x antiwrithing $ED_{50}$: morphine (56 mg/kg) and buprenorphine (0 90 mg/kg) Antinociceptive regression lines were obtained at 10 h 00 min daily. Figures in parentheses refer to 95% confidence limits.

**Table 5** Antagonism of morphine by test compounds in the mouse tail flick test

| *Dosing interval(h) before morphine $ED_{90}$* | *$AD_{50}$ (mg/kg, i.p.)* | | |
|---|---|---|---|
| | *Buprenorphine* | *Naloxone* | *Nalorphine* |
| 0.25 | 0.058(0.024–0 14) | 0.017(0.008–0.039) | 3.1(1.2–7.8) |
| 0 50 | 0 028(0.010–0.076) | 0.035(0.016–0.076) | 0.83(0.27–2.5) |
| 1.0 | 0.019(0.008–0.043) | 0.074(0.038–0.14) | 2.6(1.0–6.5) |
| 2.0 | 0.013(0.006–0.028) | 0.14(0.063–0.32) | 3.4(1.7–6.8) |
| 4 0 | 0 016(0.006–0.043) | 0.16(0.072–0.36) | 5 2(2.3–12) |

The noxious stimulus was water maintained at 55°C and the test took place 0.5 h after injection of morphine (50 mg/kg, s.c.). Figures in parentheses refer to 95% confidence limits

Antagonism was also demonstrated when mice received buprenorphine 15 min before the ED$_{90}$ dose of another narcotic agent, phenazocine (28 mg/kg). The AD$_{50}$ value of 0.035 mg/kg (0.017–0.74) was similar to that obtained against morphine. Buprenorphine was much less potent in the corresponding rat tail flick test. Thus, when buprenorphine was injected immediately before morphine, the AD$_{50}$ value at 30 min was only 2.5 mg/kg (1.3–4.5) whereas that of naloxone was 0.38 mg/kg (0.18–0.80).

Buprenorphine (0.001–10 mg/kg) was not a morphine antagonist in the rat tail pressure test; the AD$_{50}$ value for naloxone was 0.17 mg/kg (0.060–0.45). When buprenorphine (3 mg/kg) was injected either 30 or 60 min before the ED$_{50}$ dose of morphine a 100% antinociceptive effect was recorded 30 min later.

In the mouse jumping test there was no bell-shaped dose-response curve with buprenorphine when log dose was plotted against the number of mice jumping at least 6 times during 1 hour. The maximum antagonistic effect with high doses of buprenorphine (3–30 mg/kg) was only 70%, a finding in keeping with results from the mouse tail flick test. Surprisingly, buprenorphine was not an antagonist when given to morphine-dependent rats. In contrast to nalorphine (3 mg/kg), which precipitated the range of signs previously described (Bläsig, Höllt, Herz & Paschelke, 1976) and caused a significant ($P<0.05$) weight loss over 3 h, buprenorphine (0.30–10 mg/kg) induced a dose-related weight loss over 3 h which was not significantly different from control results (Table 7). Buprenorphine was clearly a morphine antagonist in non-withdrawn, morphine-dependent monkeys since a dose as low as 0.1 mg/kg precipitated a moderate abstinence syndrome with such signs as shaking, yawning and fighting. Also, when 2 drug-naïve baboons were narcotized with etorphine/methotrimeprazine (Small Animal Immobilon, Reckitt & Colman, 0.11 mg/kg, s.c.) then injected 20 min later with buprenorphine (0.1 mg/kg, i.v.) in both cases there was a rapid reversal of the narcosis.

**Table 6** Duration of morphine antagonist activities of test compounds in the mouse tail flick test

| *Compound* | *Duration (min) of effect of equi-antagonistic doses* |
|---|---|
| Buprenorphine | 966 |
| Diprenorphine | 303 |
| Naloxone | 105 |

The noxious stimulus was water maintained at 55°C. Different groups of 10 mice were challenged with morphine (50 mg/kg, s.c.) at various times after the injection of the AD$_{80}$ dose of each antagonist. The time intervals between 80% and 20% antagonism are compared for each compound.

The Straub tail ED$_{50}$ value for buprenorphine in mice was 0.16 mg/kg (0.060–0.75). It is noteworthy that pretreatment with large amounts of naloxone or diprenorphine (each at 1 and 10 mg/kg) did not significantly influence the position of the buprenorphine regression line. However, in the rat tail pressure test the buprenorphine regression line for antinociceptive effect was increasingly displaced to the right when diprenorphine (0.10, 0.30 or 1.0 mg/kg) was injected immediatley after buprenorphine (Table 8). A much larger dose of diprenorphine (e.g. 3 mg/kg rather than 0.30 mg/kg) was required to cause the same shift when this antagonist was injected 30 min after buprenorphine. It was thus more difficult to antagonize the antinociceptive effect of buprenorphine once it was well-established.

As indicated above, a bell-shaped log dose-response curve was obtained with rats in the catalepsy

**Table 7** Relative weight losses shown by morphine-pelleted rats after challenge with test compounds

| *Compound* | *Dose (mg/kg, s.c.)* | *Mean weight loss (g) (s.e. mean)* +3 h | +24 h |
|---|---|---|---|
| Saline | 5.0 ml/kg | 4.1±1.1 | 30.3±1.8 |
| Nalorphine | 3.0 | 7.0±0.94* | 28.4±1.3 |
| Buprenorphine | 0.30 | 2.9±0.37 | 26.9±1.9 |
| | 1.0 | 3.9±0.81 | 28.8±0.90 |
| | 3.0 | 4.2±0.69 | 28.4±1.9 |
| | 10 | 5.3±0.70 | 29.7±1.0 |

Groups of 8 rats were implanted with 75 mg morphine pellets on day 1 and again on day 3. The rats were weighed on day 4, test compounds were then injected and further weighings took place 3 and 24 h later.
* Significantly different ($P<0.05$) from control animals by the Mann Whitney U test.

**Table 8** The effect of diprenorphine on the antinociceptive $ED_{50}$ of buprenorphine in the rat tail pressure test

| *Treatment* | | *Buprenorphine $ED_{50}$ (mg/kg, s.c.)* | *Relative potency* |
|---|---|---|---|
| *0 min* | *0 min* | *30 min* | |
| Buprenorphine | Saline | 0.022(0 013–0.037) | 1 |
| Buprenorphine | Diprenorphine (0.10 mg/kg) | 0.34(0.16–0.73) | 17 |
| Buprenorphine | Diprenorphine (0.30 mg/kg) | 1 3(0 59–2.7) | 77 |
| Buprenorphine | Diprenorphine (1.0 mg/kg) | 6.9(4 5–10) | 313 |
| *0 min* | *30 min* | *60 min* | |
| Buprenorphine | Saline | 0 010(0.005–0.017) | 1 |
| Buprenorphine | Diprenorphine (1 mg/kg) | 0.069(0.041–0.083) | 6.8 |
| Buprenorphine | Diprenorphine (3 mg/kg) | 0 66(0.38–1.2) | 77 |

Each regression line for buprenorphine was obtained with 30 rats, either in the presence or absence of subcutaneous diprenorphine. The slopes of these lines did not differ significantly from parallelism. Figures in parentheses refer to 95% confidence limits.

experiment (Figure 2). At the time of peak effect (30 min), the maximum cataleptic response to buprenorphine (0.30 mg/kg) was only 50%. In a similar experiment with morphine the $ED_{50}$ value (at 30 min) was 10 mg/kg (6.0–18).

## Discussion

Buprenorphine is a potent antinociceptive agent with a rapid onset and long duration of action in rodents. This new narcotic antagonist analgesic is 25–40 times more potent than morphine in mouse writhing and rat tail pressure tests after subcutaneous or intraperitoneal injection and 7–10 times more potent after oral administration. The positive results obtained with buprenorphine in rodent tail flick tests are noteworthy since reference narcotic antagonist analgesics are essentially inactive when the conventional tail flick (radiant heat) method is used (Dewey, Harris, Howes & Nuite, 1970). The antinociceptive action of buprenorphine was demonstrated in mice and rats by using a modified tail flick method where water at 45, 55 or 65 °C served as the noxious stimulus (Cowan, Lewis & Macfarlane, 1971). The efficacy of buprenorphine was greater than that of pentazocine but less than that of morphine in this procedure. In contrast to the sigmoid-shaped log dose-percentage response lines obtained with morphine, the corresponding lines for buprenorphine (at 45, 55 and 65 °C) and pentazocine (at 45 °C) resembled an inverted U. On the other hand, the log dose-response relationship for buprenorphine was linear in the mouse writhing and rat tail pressure tests, that is, tests in which buprenorphine produced the maximum possible antinociceptive effect over a wide range of doses.

Biphasic dose-response curves have also been obtained with buprenorphine in tests involving different measures e.g. reduction of respiratory rate in mice or antagonism of gastrointestinal motility in rats (Cowan, Doxey & Harry, 1977); and induction of catalepsy in rats (see Results section). In these tests, and in the tail flick test, the maximum effect of buprenorphine occurred within the dose range 0.10–3.0 mg/kg. The finding that buprenorphine is less efficacious at 10 and 30 mg/kg than at 3 mg/kg in the rat tail flick test (Figure 2) might be thought due to high doses of the analgesic causing a general behavioural depression. This possibility is unlikely since the degree of catalepsy is of the same low order over the range 3–30 mg/kg in rats; moreover, it may be argued that depressed animals will take longer to react to the noxious stimulus and thus give enhanced rather than reduced, tail flick latencies.

At the receptor level, the biphasic dose-response curves obtained with buprenorphine may represent examples of non-competitive autoinhibition (Ariëns, van Rossum & Simonis, 1957). According to this theory, in certain systems buprenorphine would have an affinity not only for the so-called μ (morphine) receptor but also for a second, interdependent receptor, then as a result of an increasing interaction between buprenorphine and the second receptor, the intrinsic activity of the buprenorphine-$\mu$ complex would become correspondingly less.

Tolerance developed to the antinociceptive actions of buprenorphine in the phenylquinone-writhing test. In this respect, buprenorphine is similar to morphine but differs from cyclazocine, nalorphine and pentazocine since Smits & Takemori (1970) found that marked tolerance did not occur with these narcotic antagonist analgesics in the same writhing test. The long duration of action of buprenorphine as an agonist in this test may have contributed to the apparent divergent results. Although buprenorphine can induce tolerance in the mouse, it is known (Cowan, 1974) that a dose of naloxone as high as 10 mg/kg, subcutaneously, precipitates only a very low incidence of repetitive jumping in this species when buprenorphine is run in the primary dependence test described by Saelens, Granat & Sawyer (1971). These results provide evidence of a dissociation of physical dependence from tolerance after multiple injections of buprenorphine to mice.

The finding that buprenorphine may not produce physical dependence in patas monkeys is in agreement with the conclusion from a direct dependence study carried out in rhesus monkeys at the University of Michigan (Swain & Seevers, 1975). Also, it has recently been found that buprenorphine (10 mg $kg^{-1}$ $day^{-1}$) neither substitutes for morphine in morphine-dependent rats when the intraperitoneal infusion technique described by Teiger (1974) is used nor induces physical dependence during a 6-day infusion (50–100 mg $kg^{-1}$ $day^{-1}$) in this species (Dewey, Harris & Ritter, 1975; Dr W.L. Dewey, personal communication). These observations are at variance with the report by Martin, Gilbert, Eades, Thompson & Huppler (1975) that buprenorphine partially suppresses the withdrawal syndrome in morphine-dependent chronic spinal dogs, and that naloxone precipitates signs of abstinence in chronic spinal dogs receiving multiple intravenous injections of buprenorphine (Martin, Eades, Thompson, Huppler & Gilbert, 1976). However, it is important to note, that in the dog experiments (a) buprenorphine precipitated signs of abstinence in non-withdrawn animals (b) the slope of the regression line for buprenorphine in the suppression study was significantly less than that of morphine and d-propoxyphene and (c) the abstinence syndrome precipitated by naloxone challenge emerged rather slowly and was classified as being 'mild'. Although no explanation is available at present for the different results obtained with mice, rats and monkeys as opposed to dogs it would seem reasonable to agree with the prediction of Martin *et al.* (1976) that buprenorphine may produce no more than a liminal (and perhaps clinically insignificant) degree of physical dependence in man.

In the present work, narcotic antagonism was demonstrated with buprenorphine in mice, rats, monkeys and baboons. Compared to naloxone in the mouse tail flick test, buprenorphine was equipotent and longer acting but less efficacious. Experience with the hot water version of the tail flick test using mice has suggested that the antagonistic potencies of many analgesics, particularly oripavine-thebaine derivatives, are overestimated in relation to values obtained from other procedures. Thus, buprenorphine is approximately 7 times less potent than naloxone in the corresponding rat tail flick test and 15 times less potent in the mouse jumping test (Cowan, 1976). Buprenorphine contrasts with naloxone in the latter test by again being less efficacious and (after a slow onset) having a longer duration of action. The profile of buprenorphine as a morphine antagonist is complex since antagonism was demonstrated in rats using the tail flick test but antagonism was absent in the tail pressure test; moreover, buprenorphine precipitated signs of abstinence in non-withdrawn, morphine-dependent monkeys but not in non-withdrawn, morphine-dependent rats. The result from the tail pressure test was not entirely unexpected since experience has shown that it is difficult to detect the antagonist component of narcotic antagonist analgesics that are potent agonists in the procedure.

Relatively large doses of diprenorphine were required to antagonize the antinociceptive action of buprenorphine in the rat tail pressure test. The surprising feature of the buprenorphine-diprenorphine interaction was the critical importance of the relative timing of the two injections. Thus, a ten-fold increase in the dose of diprenorphine was required to effect the same degree of antagonism when this compound was given 30 min after buprenorphine rather than at the same time. In contrast, the corresponding antagonism of morphine by diprenorphine was only slightly affected by the same difference in injection times (Cowan & Macfarlane, unpublished observation). The greater difficulty in displacing buprenorphine, when once established, from narcotic receptors and the slow dissociation of the compound from these sites (J.M. Hambrook & M.J. Rance, personal communication) are findings reminiscent of those described for methadone on isolated tissues (Kosterlitz, Leslie & Waterfield, 1975). In this context, it may be significant that buprenorphine and methadone are the most lipophilic of the widely studied analgesics.

Psychotomimetic episodes have occurred in men after receiving high doses of many narcotic antagonist analgesics e.g. pentazocine, nalbuphine (Jasinski, Martin & Hoeldtke, 1970; Jasinski & Mansky, 1972). Dysphoric effects have not been associated with buprenorphine during extensive clinical trials involving over 1500 subjects. Since these clinical studies have also shown buprenorphine to be an effective, long-lasting and safe analgesic, it is concluded that this new agent represents a definite advance in the search for a non-psychotomimetic, narcotic antagonist analgesic of low physical dependence potential.

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# THE ANIMAL PHARMACOLOGY OF BUPRENORPHINE, AN ORIPAVINE ANALGESIC AGENT

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1 The general pharmacology of buprenorphine, a potent analgesic agent derived from oripavine, is described.

2 After acute administration of buprenorphine, the spontaneous locomotor activity of mice was increased; rats displayed stereotyped licking and biting movements; behavioural depresssion was marked in guinea-pigs but mild in rhesus monkeys. The behaviour of cats was unchanged.

3 In general, buprenorphine reduced heart rate but had no significant effect on arterial blood pressure in conscious rats and dogs.

4 In anaesthetized, open-chest cats buprenorphine (0.10 and 1.0 mg/kg, i.v.) caused no major haemodynamic changes.

5 Buprenorphine (0.01–10 mg/kg, i.a.) and morphine (0.30–30 mg/kg, i.a.) increased arterial $P\mathrm{CO}_2$ values and reduced $P\mathrm{O}_2$ values in conscious rats. With doses of buprenorphine greater than 0.10 mg/kg (a) the duration of respiratory depression became less, (b) ceiling effects occurred such that the maximum effects produced were less than those obtained with morphine.

6 Buprenorphine was a potent and long-lasting antagonist of citric acid-induced coughing in guinea-pigs.

7 At a dose level 20 times greater than the $\mathrm{ED}_{50}$ for antinociception (tail pressure), morphine suppressed urine output to a greater extent than the corresponding dose of buprenorphine in rats.

8 Over the range 0.01–1.0 mg/kg (s.c.), buprenorphine slowed the passage of a charcoal meal along the gastrointestinal tract in rats. After doses in excess of 1 mg/kg, the meal travelled increasingly further such that the distances measured after 10 and 30 mg/kg did not differ significantly from control values. In contrast, the morphine dose-response relationship was linear.

## Introduction

Buprenorphine [*N*-cyclopropylmethyl-7*α*-(1-*S*-hydroxy, 1,2,2-trimethylpropyl)-6,14-*endo*ethano-6,7,8, 14-tetrahydronororipavine](RX 6029-M) is a new potent antinociceptive agent, chemically related to the narcotic antagonist, diprenorphine (M5050). It has been predicted from studies in rodents and monkeys that buprenorphine will be a long-acting, effective analgesic with a low physical dependence liability in man (Cowan, Lewis & Macfarlane, 1977). In the present paper, a comparison is made of the effects of buprenorphine and reference analgesics on animal behaviour, cardiovascular and respiratory function, experimental cough, urine output and gastrointestinal motility. A preliminary communication on the general pharmacology of buprenorphine was given at the Sixth International Congress of Pharmacology, Helsinki, Finland (July, 1975).

## Methods

### Animals

The experiments were carried out on male albino mice (MFI/Ola, 18–24 g), male Sprague-Dawley albino rats in the weight ranges indicated in the text, male albino guinea-pigs (250–350 g), cats (2–3 kg), beagle dogs (8–15 kg) and rhesus monkeys (*Macaca mulatta*, 4–6 kg).

### Compounds

The following compounds were dissolved or diluted in 0.9% w/v NaCl solution (saline) and the doses calculated in terms of the free base: buprenorphine hydrochloride (mol. wt. of base is 467.6; Reckitt & Colman), butorphanol tartrate (Bristol), methadone hydrochloride, B.P. (Macfarlan Smith), morphine sulphate, B.P. (Macfarlan Smith), naloxone hydrochloride (Endo) and pentazocine lactate (Sterling-Winthrop). The free base of cyclazocine (Sterling-Winthrop) was dissolved in a minimal amount of 0.1 N HCl, the pH adjusted to 5 with $NaHCO_3$ solution and made up to volume with saline.

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*Behavioural effects*

Groups of 10 mice were given 3 x 3 min training runs over 1 h on a rota-rod treadmill (Ugo Basile); they were injected subcutaneously with buprenorphine or cyclazocine, then retested 30 or 60 min later. Muscular incoordination was considered present in those mice falling off the rota-rod within 30 seconds.

Groups of 5 mice or 3 rats (100–120 g) received saline (s.c.) at 10 h 00 min and spontaneous locomotor activity in the home (makrolon) cage was recorded at 15 min intervals for the following 6 h with an Animex monitor (Farad Electronics, sensitivity and tuning set at 40 µA). The same animals were injected with test drug 24 h later and the cumulative activity counts were again recorded.

The cataleptic effects of buprenorphine and morphine were estimated 0.5, 1.5 and 2.5 h after subcutaneous administration to groups of 10 guinea-pigs. The number of animals leaving both hind legs over a horizontal metal rod (4 cm above bench level) for longer than 45 s was recorded.

The behaviour of 5 cats (grouped in a large animal room) was monitored for 6 h after subcutaneous injection of saline. One week later each cat received buprenorphine subcutaneously and the animals were observed over the following 6 h and at regular intervals throughout the following day.

Rhesus monkeys (in two groups of 3 and 2) whose behavioural patterns had previously been recorded were injected subcutaneously with buprenorphine and observed through a one-way mirror for the following 6 hours.

*Cardiovascular effects*

The descending aorta of rats (200–250 g) was cannulated as described by Weeks & Jones (1960). Three days later each animal was placed in a restraint cage and the cannula was connected to a pressure transducer which, in turn, was linked to a polygraph (Physiograph, E & M Instrument Co.) via a preamplifier. Two control measurements of blood pressure and heart rate were recorded, heart rate being measured directly from the trace by counting beats over a 10 s period. Groups of at least 4 rats were injected intraperitoneally with either saline or test compound and the effects were then monitored for 3 hours.

Cats were anaesthetized with a halothane/oxygen mixture. The femoral vein was cannulated and drugs were injected by this route. Anaesthesia was maintained with chloralose (75 mg/kg, i.v.). Artificial respiration with an Ideal respiration pump (20 strokes/min, 10 ml/kg room air) was started immediately the thorax was opened. Mean aortic blood flow in the brachiocephalic artery and the descending aorta was monitored with 2 electromagnetic flow probes linked via a twin-channel blood flowmeter (Biotronex) to 2 separate potentiometric recorders. The left subclavian artery was used to record blood pressure. A strain guage was sutured to the wall of the right ventricle. All recordings were displayed on a polygraph (Hewlett Packard 7700). The following parameters were measured or derived: myocardial force of contraction, heart rate, mean arterial blood pressure, cardiac output (cardiac index), stroke volume (stroke index) and peripheral resistance (mean arterial pressure, mmHg/cardiac index, $l\ min^{-1}\ m^{-2}$). The surface area ($m^2$) of the cat was calculated from the formula, $K = area\ (cm^2)/weight\ (g)^{2/3}$, using Thomas' constant for the cat (K = 10) (Altman & Dittmer, 1964).

Heart rate and blood pressure were monitored in beagle dogs trained to stand in a Pavlovian sling. An electrosphygmograph (E & M Instrument Co.) was used to record (indirectly) systolic and diastolic blood pressure. The electrosphygmograph combines a pressure transducer and a preamplifier to produce single-channel recordings of occluding cuff pressure and superimposed Korotkoff sounds. A wrap-round cuff with attached microphone (E & M 93-40074) was used to monitor blood pressure from the right foreleg. Drugs were injected via the cephalic vein. Respiratory rate was counted visually.

*Respiratory effects*

Control rates of respiration of mice were measured with a pressure transducer coupled to an instant ratemeter. Groups of 10 animals were then injected subcutaneously with either saline or test compound and the rates were again measured 30 min later.

The descending aorta of rats (200–300 g) was cannulated as described by Weeks & Jones (1960). Two days later the animals were placed in individual restraint cages. Two arterial blood samples were taken from each rat and immediately analysed for $P_{CO_2}$ and $P_{O_2}$ with a Blood Gas Analyser (Model 48C, Electronic Instruments Ltd.). Test compounds or saline were then given (1 ml/kg, i.a.) and further measurements of $P_{CO_2}$ and $P_{O_2}$ were taken 15,30 and 45 min later.

*Antitussive effects*

Groups of 15 guinea-pigs received oral injections of either distilled water or test compound; 30 min later each animal was individually confined in a test chamber and exposed to an atmosphere of citric acid. This was delivered as an aerosol containing approximately 4 mg citric acid/l of air, at a flow rate of

7.5 litres/minute. After an equilibration time of 3 min, the incidence of coughing was monitored over the following 5 min using pressure transducers connected to a polygraph (Van Gogh). One week later the animals were retested on a cross-over basis. The reduction in the number of coughs at each dose level was assessed by comparison with the controls. The dose required to inhibit the number of coughs by 50% was estimated. Approximate durations of action were obtained by noting the time taken for the antitussive effect, caused by an $ED_{50}$ dose, to return to control values in groups of 15 guinea-pigs.

### *Effect on urine output*

Rats (120–140 g) were deprived of food for 18 h but allowed water until 1 h before the test. Groups of 8 animals were then water-loaded (3 ml/100 g, orally), injected subcutaneously with saline or test compound and placed in metabolism cages. Urine volumes were recorded every 30 min for 6 hours. Each group received the same treatment on a further 4 occasions. A minimum of 72 h elapsed between successive treatments.

### *Effect on gastrointestinal propulsion*

Groups of 10 rats (120–140 g) were deprived of food for 18 h but allowed water until 1 h before the test. Saline or test drug was administered subcutaneously or orally and a charcoal meal (5 ml/kg; Green, 1959) was given by stomach tube 20 min (s.c.) or 50 min (orally) later. A further 10 min later each rat was killed by cervical dislocation. The distance travelled by the meal along the small intestine from the pyloric sphincter was measured and calculated as a percentage of the total length.

### *Acute toxicity*

Acute median lethal doses were estimated 7 days after intravenous, intraperitoneal, subcutaneous and oral administration of buprenorphine to groups of 10 male and female mice (18–22 g) and rats (60–80 grams).

### *Statistical evaluation*

Quantal data were subjected to logit analyses using Bliss 17, a computer programme written by Professor D.J. Finney (see Finney, 1971). Figures in parentheses in the Results section refer to the 95% confidence limits of the $ED_{50}$ or $LD_{50}$.

## Results

The $ED_{50}$ value for buprenorphine in the mouse rotarod test was >100 mg/kg; an optimal value of 3.2 mg/kg (1.6–4.9) was obtained with cyclazocine at 30 minutes.

Buprenorphine (0.10, 0.30 and 1.0 mg/kg) increased the spontaneous locomotor activity of groups of 5 mice in relation to control sessions with the same animals. High doses of naloxone, when injected 5 min before a standard dose of buprenorphine (0.10 mg/kg), antagonized the potentiating effects of the latter compound in a dose-related manner (Table 1). Although buprenorphine (0.10–3.0 mg/kg) increased the spontaneous locomotor activity of groups of 3 rats over 6 h sessions, the animals initially remained immobile and adopted a characteristic hunchbacked ('hedgehog') posture; preening and huddling (Giurgea & Van Kemeulen, 1973) were absent. The rats reacted normally to auditory and tactile stimuli. Repetitive licking and biting of the limbs and the cage, and occasionally fighting, occurred mainly 4–5 h after injection of buprenorphine.

Both buprenorphine and morphine produced catalepsy in guinea-pigs. The logit lines did not differ significantly from parallelism; at the time of peak effect (30 min) the $ED_{50}$ values were 0.007 mg/kg (0.003–0.015) and 2.2 mg/kg (1.2–3.8), respectively.

**Table 1** The effect of naloxone on increases in locomotor activity caused by buprenorphine in mice

| *Treatment* | | *Mean activity counts* |
|---|---|---|
| *–5 min (i.p.)* | *0 min (s.c.)* | |
| Saline | Saline | 3346 |
| Naloxone (10 mg/kg) | Saline | 3281 |
| Naloxone (30 mg/kg) | Saline | 3671 |
| Naloxone (100 mg/kg) | Saline | 2660 |
| Saline | Buprenorphine (0.10 mg/kg) | 12560 |
| Naloxone (10 mg/kg) | Buprenorphine (0 10 mg/kg) | 8712 |
| Naloxone (30 mg/kg) | Buprenorphine (0.10 mg/kg) | 5775 |
| Naloxone (100 mg/kg) | Buprenorphine (0.10 mg/kg) | 3612 |

Total counts were obtained from 2 different groups of 5 mice run between 10 h 00 min and 16 h 00 min.

Buprenorphine (0.50, 1.0, 2.0, 5.0 and 10 mg/kg) did not alter the behaviour of 5 cats and morphine-like mania did not occur. Mydriasis was observed at all dose levels and occasional bursts of running were noted with the highest dose.

After injection into 5 drug-naïve rhesus monkeys, buprenorphine (0.03, 0.10, 0.30, 1.0 and 3.0 mg/kg) caused only a slight behavioural depression (eyes closing momentarily and dazed facial expressions) which lasted about 40 min and which was not dose-related. The animals reacted normally to environmental stimuli at all times.

In normotensive, conscious rats the two lower doses of buprenorphine (0.10 and 1.0 mg/kg, i.p.) reduced the heart rate, whereas the top dose (10 mg/kg) had the opposite effect. At a dose level of 10 mg/kg, morphine caused a marked bradycardia 0.5 and 1.0 h after injection (Table 2). A general trend of higher blood pressure readings occurred in the same rats after administration of saline or the analgesics. Thus,

**Table 2** The effect of test compounds on the heart rate and blood pressure of conscious rats

| *Compound* | *Dose (mg/kg, i.p.)* | n | *Mean heart rate (s.e. mean) (beats/min)* | | | | |
|---|---|---|---|---|---|---|---|
| | | | Pre-drug | 0.5 h | 1 h | 2 h | 3 h |
| Saline | 10 ml/kg | 8 | 403 ± 8 | 404 ± 27 | 411 ± 17 | 404 ± 13 | 443 ± 13 |
| Morphine | 1.0 | 4 | 406 ± 10 | 375 ± 24 | 403 ± 15 | 400 ± 11 | 423 ± 25 |
| | 10 | 4 | 411 ± 11 | 298 ± 10*** | 368 ± 10*** | 423 ± 15 | 428 ± 25 |
| Buprenorphine | 0.10 | 9 | 434 ± 9 | 411 ± 20 | 404 ± 16 | 416 ± 31 | 423 ± 33 |
| | 1.0 | 9 | 432 ± 12 | 374 ± 13* | 418 ± 13 | 419 ± 15 | 433 ± 16 |
| | 10 | 9 | 408 ± 8 | 408 ± 7 | 429 ± 12 | 441 ± 12** | 423 ± 13 |
| | | | *Mean arterial blood pressure (s.e. mean) (mmHg)* | | | | |
| Saline | 10 ml/kg | 8 | 122 ± 3 | 127 ± 4 | 134 ± 4** | 129 ± 4** | 135 ± 5* |
| Morphine | 1.0 | 4 | 122 ± 3 | 125 ± 4 | 130 ± 7 | 119 ± 4 | 124 ± 5 |
| | 10 | 4 | 123 ± 3 | 131 ± 4 | 144 ± 3* | 133 ± 3* | 125 ± 3 |
| Buprenorphine | 0.10 | 9 | 130 ± 4 | 138 ± 6* | 139 ± 5** | 137 ± 5* | 135 ± 6 |
| | 1.0 | 9 | 129 ± 4 | 137 ± 6** | 136 ± 5* | 142 ± 8* | 136 ± 6* |
| | 10 | 9 | 125 ± 2 | 134 ± 4* | 134 ± 4* | 129 ± 4 | 128 ± 6 |

*$P < 0.05$; **$P < 0$ 01, ***$P < 0.001$ (Student's *t* test for paired data)

**Table 3** The effect of test compounds on the heart rate and blood pressure of conscious dogs

| *Compound* | *Dose (mg/kg, i.v.)* | n | *Mean heart rate (s.d.) (beats/min)* | | | |
|---|---|---|---|---|---|---|
| | | | Pre-drug | + 5 min | + 15 min | + 30 min |
| Saline | 0.10 ml/kg | 3 | 123 ± 6 | 117 ± 2 | 107 ± 6* | 116 ± 7 |
| Buprenorphine | 0.01 | 3 | 121 ± 5 | 112 ± 24 | 113 ± 29 | 114 ± 24 |
| | 1.0 | 3 | 115 ± 9 | 80 ± 10* | 80 ± 8* | 80 ± 0* |
| Morphine | 0.10 | 3 | 113 ± 6 | 117 ± 6 | 113 ± 6 | 107 ± 15 |
| | 10 | 3 | 123 ± 6 | 91 ± 36 | 92 ± 39 | 92 ± 39 |
| | | | *Mean arterial blood pressure (s.d.) (mmHg)* | | | |
| Saline | 0.10 ml/kg | 3 | 103 ± 3 | 105 ± 8 | 117 ± 6* | 116 ± 5 |
| Buprenorphine | 0 01 | 3 | 129 ± 20 | 113 ± 21 | 124 ± 24 | 127 ± 31 |
| | 1.0 | 3 | 109 ± 20 | 121 ± 7 | 112 ± 10 | 111 ± 7 |
| Morphine | 0.10 | 3 | 110 ± 13 | 106 ± 7 | 93 ± 3 | 100 ± 10 |
| | 10 | 2 | 100 | 99 | 90 | 96 |

* $P < 0.05$ (Student's *t* test for paired data).

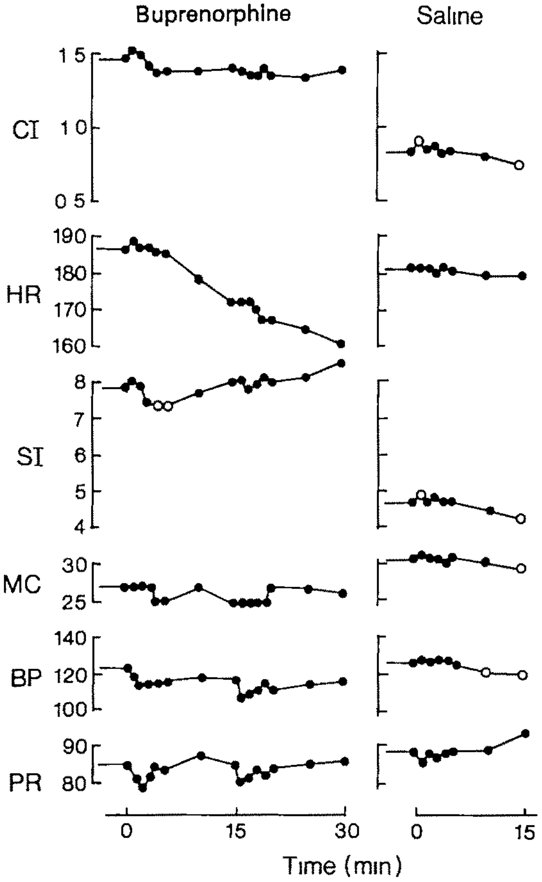

**Figure 1** The haemodynamic effects of buprenorphine and saline in open-chest cats anaesthetized with chloralose (75 mg/kg, i.v.). Buprenorphine was injected at 0 min (0.10 mg/kg) and again at 15 min (1.0 mg/kg) into the femoral veins of 3 cats. Saline (1.0 mg/kg, i.v.) was injected at 0 min into 5 different cats. Mean values represented by open circles are significantly different from initial values ($P<0.05$, Student's $t$ test for paired data). CI, cardiac index (l $\text{min}^{-1}$ $\text{m}^{-2}$); HR, heart rate (beats/min); SI, stroke index (ml $\text{beat}^{-1}$ $\text{m}^{-2}$); MC, myocardial force of contraction (g); BP, mean arterial blood pressure (mmHg); PR, peripheral resistance (arbitrary units).

the statistically significant values for buprenorphine should be interpreted with caution since saline induced a similar rise in blood pressure over the 3 h session.

In anaesthetized, open-chest cats buprenorphine (0.10 and 1.0 mg/kg, i.v.) caused no major haemodynamic effects (Figure 1). The observed bradycardia did not reach a statistically significant level probably because a group size of only 3 cats was used.

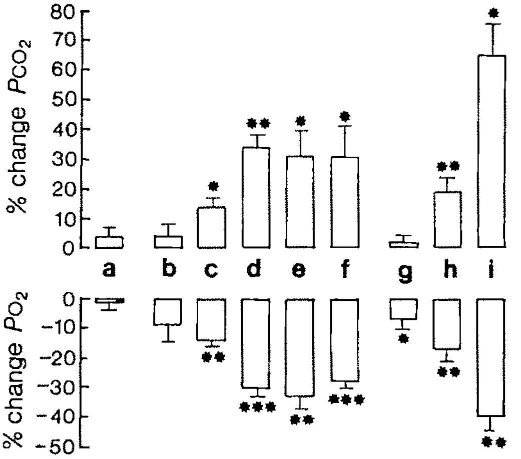

**Figure 2** The mean percentage change in arterial $P\text{CO}_2$ and $P\text{O}_2$ values 15 min after (a) intra-arterial injection of saline (1 ml/kg), (b–f) buprenorphine (0.01, 0.03, 0.10, 1.0 and 10 mg/kg) or (g–i) morphine (0.30, 3.0 and 30 mg/kg) to groups of 5 conscious rats. Vertical lines show s.e. means. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ (Student's $t$ test for paired data).

A high dose of buprenorphine (1 mg/kg, i.v.) significantly decreased the heart rate of conscious dogs but had no marked effect on arterial blood pressure (Table 3). At this dose level all 3 dogs began to pant and showed head-nodding movements. Immediately after a high dose of morphine (10 mg/kg, i.v.), the dogs displayed 'sham rage' for about 15 s (Domer & Josselson, 1964) and then collapsed. It was difficult to monitor the blood pressure with an electrosphygmograph in these animals since the pulse was so weak; indeed, in one dog a blood-pressure trace could not be obtained.

The respiratory rate of mice was decreased in a dose-related manner by morphine (1,5 and 10 mg/kg, s.c.); the top dose caused a 56% reduction ($P<0.001$, Student's $t$ test for paired data). The maximum effect with buprenorphine (0.001–10 mg/kg, s.c.) occurred after 0.10 mg/kg when a 22% reduction was recorded ($P=0.02$).

Buprenorphine (0.01–10 mg/kg, i.a.) and morphine (0.30–30 mg/kg, i.a.) increased arterial $P\text{CO}_2$ values and reduced $P\text{O}_2$ values at 15 min in conscious rats (Figure 2). With buprenorphine, depression of respiration reached a plateau over the dose-range 0.10–10 mg/kg. Interestingly, the duration of respiratory depression became less as the dose of buprenorphine was increased; thus, after 10 mg/kg, the $P\text{CO}_2$ and $P\text{O}_2$ values had returned to control levels at 45 minutes. In contrast, the response to morphine increased linearly with dose and showed no signs of recovery at 45 minutes.

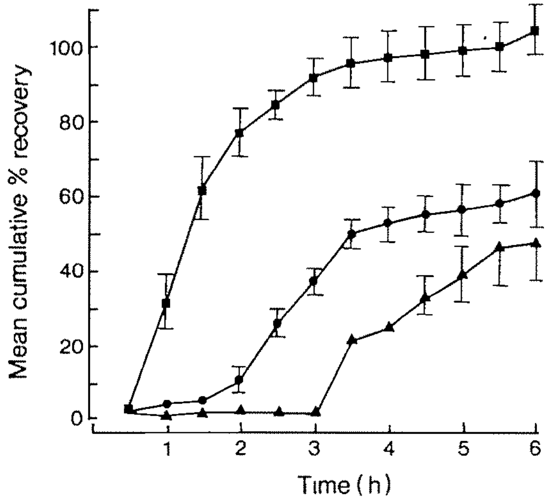

**Figure 3** Effect of saline (■), buprenorphine (0.48 mg/kg, s.c. ●) or morphine (35 mg/kg, s.c. ▲) on the urine output of groups of 6 rats over 6 hours Each group received the same treatment on 5 occasions. The mean cumulative percentage recovery of the oral water-load (3 ml/100 g) is shown on the ordinate scale. Vertical lines show s.e. means

Buprenorphine was more potent and longer acting than either butorphanol or methadone as an oral antitussive agent in guinea-pigs (Table 4).

The doses of buprenorphine (0.48 mg/kg) and morphine (35 mg/kg) chosen for the urine output study were each 20 times higher than the antinociceptive (tail pressure) $ED_{50}$ values obtained with the same batch of rats. Both analgesics suppressed urine output. The time taken to recover 40% of the water load was 1.25 h after injection of saline and 3.25 and 5.25 h in the case of buprenorphine and morphine, respectively (Figure 3).

After subcutaneous injection, buprenorphine (1 mg/kg) and morphine (3 mg/kg) were essentially equi-efficacious in slowing the passage of a charcoal meal along the small intestine of rats. Doses of buprenorphine within the range 0.01–1.0 mg/kg progressively reduced the distance travelled by the meal.

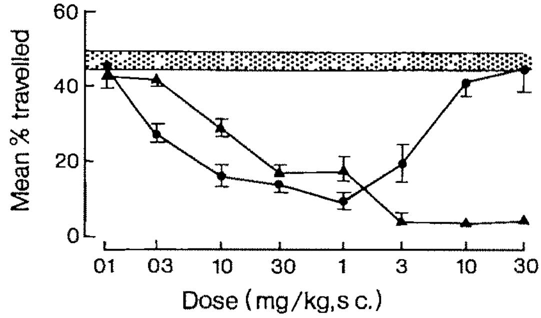

**Figure 4** The effect of buprenorphine (●) and morphine (▲) on intestinal motility in rats ($n = 10$) Each point gives the mean distance travelled by a charcoal meal expressed as a percentage of the total length of the small intestine. Vertical lines show s.e means The stippled band represents the mean percentage distance (with s.e. mean) travelled by the meal in 30 control rats.

On the other hand, after doses in excess of 1 mg/kg, the meal travelled increasingly further such that the distances measured after 10 and 30 mg/kg did not differ significantly from control values. This phenomenon did not occur with morphine (Figure 4). After oral administration, the dose-response curve of buprenorphine (0.001–100 mg/kg) was also curvilinear (U-shaped); the maximum constipative effect was again associated with a dose level of 1 mg/kg but this was significantly less than that obtained with high doses of morphine (30 and 100 mg/kg).

The median acute lethal doses of buprenorphine after intravenous, intraperitoneal and oral administration are presented in Table 5. Most deaths occurred within 24 h of injection and were a consequence of respiratory depression. Although $LD_{50}$ values were computed after intraperitoneal administration to both mice and rats, values after subcutaneous injection could not be obtained even after doses of 300 mg/kg to mice and 600 mg/kg to rats.

The value of the $LD_{50}$ for morphine (i.p.) in male rats in this laboratory in the past was found to be 306 mg/kg (237–395) and therefore the therapeutic

**Table 4** The antitussive effects of test compounds in guinea-pigs

| *Compound* | *$ED_{50}$ (mg/kg, orally)* | *Potency ratio* | *Length of action (h)** |
|---|---|---|---|
| Buprenorphine | 0.067 (0 019–0.22) | 31 (11–87) | >8 <12 |
| Butorphanol | 0.12 (0.039–0 26) | 19 (8–47) | >0.5 <1 |
| Methadone | 3.6 (1 3–6.6) | 1.0 | >2 <4 |

Groups of 15 guinea-pigs were individually exposed for 5 min to a citric acid aerosol, 30 min after administration of drugs or water.

* The time taken for the antitussive effect, caused by the $ED_{50}$ dose, to return to control values ($P < 0.05$, Student's *t* test for paired data).

index ($LD_{50}/ED_{50}$ in the rat tail pressure test) is 464. The corresponding index for buprenorphine is 12,313, representing a greatly increased margin of safety.

**Table 5** Acute toxicities of buprenorphine in mice and rats

| | | *Mouse* | *Rat* |
|---|---|---|---|
| *Sex* | *Route* | *$LD_{50}$ (mg/kg)* | $LD_{50}$ (mg/kg) |
| M | i v | 24 (21–27) | 38 (28–51) |
| F | i.v | 29 (26–31) | 31 (26–37) |
| M | i p. | 97 (84–112) | 197 (145–277) |
| F | i p. | 90 (65–125) | 207 (168–255) |
| M | oral | 261 (234–291) | >600 |
| F | oral | 260 (233–304) | >600 |

## Discussion

Buprenorphine resembled morphine by increasing spontaneous locomotor activity in mice and by causing catalepsy in guinea-pigs. In contrast to the curvilinear dose-effect realtionship observed for catalepsy with buprenorphine in rats (Cowan, Lewis & Macfarlane, 1977), catalepsy was still maintained in guinea-pigs after high doses (0.10–10 mg/kg) of this compound. According to Fog (1970) and Costall & Naylor (1974), morphine does not induce stereotyped behavioural patterns in rats after a single subcutaneous injection but does so in rats tolerant to morphine. Buprenorphine, therefore, differs from morphine but resembles the long-acting analgesic (–)-α-acetylmethadol (Henderson & Westkaemper, 1975), since it caused a stereotyped behavioural syndrome in rats (licking and biting of the cage, mutilation of the limbs) which was optimal about 4 h after *acute* administration. Effects such as catalepsy and stereotypy, as well as results from other behavioural studies (Cowan, Dettmar & Walter, 1975a,b), are indicative of buprenorphine interacting with central dopaminergic systems. This suggestion is supported by the finding that buprenorphine (3 and 10 mg/kg, s.c.) significantly increases the content of homovanillic acid (but not that of 5-hydroxyindoleacetic acid or 3-methoxy-4-hydroxyphenylglycol) in the forebrains of rats (Cowan, Dettmar & Walter, 1976).

Buprenorphine differs from morphine in causing no mania in cats and no deep ('narcotic') depression in monkeys. Unlike cyclazocine (Villarreal, 1972), buprenorphine does not cause muscular incoordination and ataxia in mice and monkeys. Moreover, the bizarre behavioural signs elicited by cyclazocine in rats, and thought to be indicative of psychotomimetic potential (Schneider, 1968), are not observed after administration of buprenorphine.

A reduction in heart rate was generally observed after the administration of buprenorphine to conscious rats and dogs and to anaesthetized, artificially respired cats. Relative to control values, the analgesic had no significant effect on arterial blood pressure in these species. In the study with dogs, a high intravenous dose of buprenorphine caused unusual head-nodding movements while 'sham rage', salivation, urination, defaecation and narcosis characterized the morphine-syndrome. Vomiting was not associated with either drug. Buprenorphine did not produce effects in open-chest cats that would contraindicate its use in the clinic. Thus, after doses of 0.10 and 1.0 mg/kg intravenously there was no evidence of depressed myocardial contractility while the cardiac index, stroke index and peripheral resistance were essentially unchanged.

Buprenorphine and morphine both caused respiratory depression in conscious rats. However, there were potentially important differences in the character of the respiratory depression. First, the $P\text{CO}_2$ and $P\text{O}_2$ dose-response curves of buprenorphine were shallower than those of morphine. Second, ceiling effects occurred with increasingly larger doses of buprenorphine such that the maximum effects produced were less than those obtained with morphine. Finally, it is of interest that respiratory depression caused by the highest dose of buprenorphine tested (10 mg/kg, i.a.) was of shorter duration than that induced by morphine (0.30–30 mg/kg, i.a.).

Buprenorphine caused a dose-related decrease in the frequency of (chemically-induced) coughing in unanaesthetized guinea-pigs. The oral potency of buprenorphine as an antitussive agent and its duration of action compare favourably with that of methadone and the recently introduced narcotic antagonist analgesic, butorphanol. The latter compound is, in fact, one of the most potent cough suppressants of its type (Cavanagh, Gylys & Bierwagen, 1976).

Buprenorphine resembled morphine in suppressing urine output in rats loaded with water. Additional experiments are necessary to establish whether or not buprenorphine has a direct renal effect and/or stimulates the release of antidiuretic hormone.

Morphine delays gastric emptying time and/or decreases propulsive contractions of the small intestine of rats in a linearly-related manner (Figure 4). The effect of buprenorphine on the transit of a test meal does not show a simple relationship to dose. The plot of log dose against response is U-shaped rather than linear and is reminiscent of the curvilinear relationship obtained with buprenorphine in certain rodent antinociceptive tests, the rat catalepsy test (Cowan, Lewis & Macfarlane, 1971; 1977) and on respiratory rate in mice (see Results section). In view of the qualitatively different dose-response lines obtained for buprenorphine and morphine in the present experiments, it is not possible to compare potencies of the two analgesics in causing constipation. However, the results have shown that morphine is more efficacious than buprenorphine as a constipative agent after oral administration while maximal effects are of the same order after subcutaneous administration. At the receptor level, it would be of interest to know if the shape and position of the buprenorphine dose-response curve alters when rats are pretreated with either naloxone or methysergide (Burks & Long, 1967) or receive multiple injections of buprenorphine. Experiments designed to clarify these points are now in progress.

It is a pleasure to thank Mr T.L. Berridge and Mr J.J. Preece for expert technical assistance. Generous gifts of butorphanol (Bristol), cyclazocine (Sterling-Winthrop) and naloxone (Endo) are gratefully acknowledged.

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# LIGATED RENAL PEDICLES AND DURATION OF ACTION OF NEOSTIGMINE AND PYRIDOSTIGMINE

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1 The duration of neostigmine or pyridostigmine antagonism of a (+)-tubocurarine (Tc) neuromuscular blockade was determined in the cat anterior tibialis-peroneal nerve preparation with and without ligated renal pedicles.

2 The infusion rate of Tc required to maintain a 90% depression of twitch tension was reduced from 8.8 ± 1.4 (s.e.) to 3.4 ± 0.6 $\mu g\ kg^{-1}\ min^{-1}$ by renal pedicle ligation.

3 Renal pedicle ligation resulted in an increased duration of antagonism of Tc by both neostigmine and pyridostigmine.

## Introduction

Acetylcholinesterase inhibitors commonly are administered to antagonize the neuromuscular blockade produced by (+)-tubocurarine (Tc). The blockade from Tc rarely outlasts inhibition of acetylcholinesterase by neostigmine (Walts, Thorpe & Dillon, 1971). However, recent clinical reports suggest that the block may reappear when Tc presence is prolonged by the absence of renal function (Gibaldi, Levy & Hayton, 1972; Miller & Cullen, 1976). This implies that the absence of renal function does not equally influence the persistence of Tc and of neostigmine. In a separate study, the plasma half life of pyridostigmine, but not that of neostigmine, was prolonged in rats with ligated renal pedicles (Burdfield & Calvey, 1973). However, they did not correlate the plasma concentrations of neostigmine or pyridostigmine with inhibition of acetylcholinesterase or antagonism of Tc. Since pyridostigmine normally has a longer duration of action than neostigmine when antagonizing Tc (Miller, Van Nyhuis, Eger, Vitez & Way, 1974), the greater prolongation of pyridostigmine's presence by renal failure suggests it should be used, rather than neostigmine, to antagonize Tc during renal failure. We tested whether, indeed, absence of renal function differed in its effect on the durations of action of pyridostigmine and neostigmine when antagonizing a Tc neuromuscular blockade.

## Methods

Thirty-one cats, 1.8 to 3.5 kg, were anaesthetized with chloralose, 60 mg/kg, and urethane, 500 mg/kg, intraperitoneally. Ventilation was controlled with a Harvard volume ventilator through a tracheostomy. The tendon of the anterior tibial muscle was freed, sectioned near its attachment, and connected to a Grass FT. 03 force-displacement transducer. The sciatic nerve was sectioned. The distal peroneal nerve was isolated and supramaximal stimuli (2 to 10 V) of 0.3 ms duration and 0.1 Hz were applied from a Grass stimulator (Model S4G) through shielded platinum electrodes to stimulate the muscle indirectly. The resulting force of muscle contraction was recorded continuously on a polygraph as discrete twitches in such a manner that twitch tension was proportional to isometric contractile force. In 17 of the 31 cats studied, the abdomen was opened, and both renal pedicles carefully identified and ligated.

A bolus intravenous injection of Tc, 0.2 mg/kg, then was given, after which Tc, 100 μg/ml, was infused from a Harvard pump at a rate that produced a constant 90% depression of twitch tension. The rate of infusion necessary decreased progressively for the next 15–45 min, after which it remained constant, as determined by at least 15 min of observation. Details of this constant-infusion technique have been described (Miller *et al.*, 1974). While the Tc infusion was continued at this rate, neostigmine or pyridostigmine was adminsitered as an intravenous bolus. Atropine, (10 μg/kg, i.v.) was given before neostigmine or pyridostigmine to prevent bradycardia and hypotension. The resultant maximum antagonism of twitch depression was calculated as a percentage of the pre-existing 90% depression (e.g., a peak rise to 40% of the pre-Tc twitch would be calculated as (40–10) 100/90, or 33% antagonism (Figure 1) (Miller *et al.*, 1974)). In addition, we measured times from neostigmine or pyridostigmine administration to peak effect (onset time) and to 50% return to the Tc-depressed twitch height (duration of action) (Figure 1). Subsequent doses of neostigmine or pyridostigmine

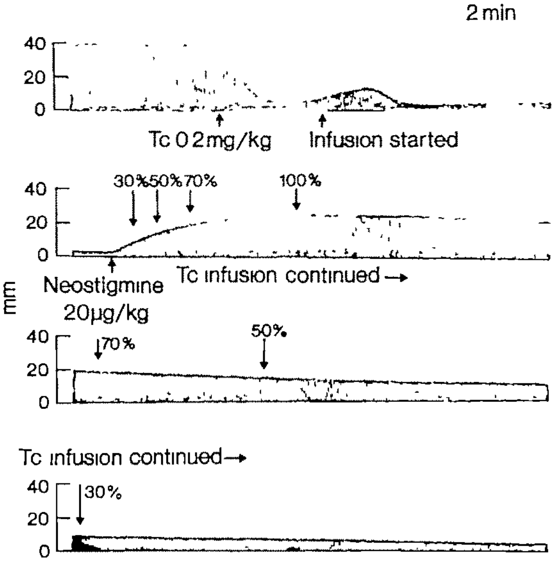

**Figure 1** Example of one of the cat neuromuscular studies. After the infusion rate of (+)-tubocurarine (Tc) which produced constant 90% depression of twitch tension had been established, neostigmine was administered Times from neostigmine administration to 30, 50, 70 and 100% of peak effect and 70, 50 and 30% to return of the Tc-depressed twitch can be determined.

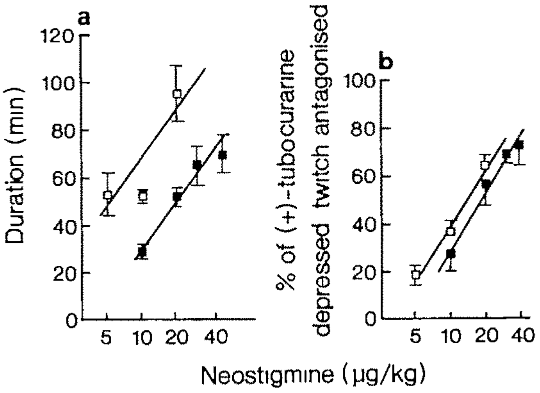

**Figure 2** Correlation between dose of neostigmine (plotted on a log scale) and (a) duration of antagonism of the (+)-tubocurarine depressed twitch and (b) % of (+)-tubocurarine-depressed twitch antagonized. Duration was defined as time from neostigmine administration of 50% return to the (+)-tubocurarine depressed twitch tension. (■) Control, (□) ligated renal pedicles; each symbol represents the mean, vertical lines show s.e. mean. Ligation of renal pedicles significantly increased the duration of neostigmine action. The number of cats studied at each dose can be obtained from Figure 4.

were given after the twitch had returned to 90% depression for at least 15 minutes.

All the above values were compared in cats with and without ligation of renal pedicles which was performed before initiation of the Tc infusion. Arterial $CO_2$ tensions, pH, and rectal temperatures were maintained between 30–40 torr, 7.30–7.41, and 36–38°C respectively.

In four additional cats with ligated renal pedicles, Tc, 1.0 mg/kg, was administered as an intravenous bolus. Thirty minutes later neostigmine, 40 µg/kg, was administered intravenously. Twitch tension then was observed for 6 hours.

Analysis of covariance was used for part of the statistical analyses. Linear regression analysis and unpaired *t* tests were carried out for the remaining results.

## Results

Ligation of renal pedicles did not alter twitch tension or arterial blood pressure. There was no difference in resting twitch tension between cats with and without ligated renal pedicles. However, ligation did reduce the infusion rates required to maintain a constant 90% depression of twitch tension from $8.9 \pm 1.4$ to $3.4 \pm 0.6$ µg $kg^{-1}$ $min^{-1}$ ($P < 0.01$). Accordingly, less neostigmine and pyridostigmine were required to antagonize the Tc depressed twitch during ligation of renal pedicles. Ligation of renal pedicles also reduced the $ED_{50}$ of neostigmine and pyridostigmine (dose which resulted in a 50% antagonsim of Tc-depressed twitch) from 17 to 13.5 ($P < 0.05$) and from 145 to 78 µg/kg ($P < 0.01$) respectively (Figures 2 and 3). The neostigmine curves did not deviate from parallelism (Figure 2). Although the pyridostigmine curves appear to be nonparallel, their deviation is of marginal significance ($P < 0.10 > 0.05$) (Figure 3).

By analysis of variance or unpaired *t* test, the onset times were not affected by ligation of the renal pedicles (Figure 4) with one exception. By an unpaired *t* test the onset times with the 300 µg/kg dose of pyridostigmine were different (Figure 4) ($P < 0.01$). By analysis of covariance, the durations of action of both neostigmine and pyridostigmine were prolonged by ligation ($P < 0.01$) (Figures 2 and 3). Comparison of the individual doses was performed by an unpaired *t* test. The duration of action of 5 and 10 µg/kg doses of neostigmine in cats with ligated pedicles was longer than the 10 µg/kg dose in cats without ligation ($P < 0.01$) (Figure 2). The 20 µg/kg dose of neostigmine in cats with ligated pedicles had a longer duration of action than either the 20, 30, or 40 µg/kg dose in the cats without ligation ($P < 0.05$) (Figure 2). The 50 and 100 µg dose of pyridostigmine had a

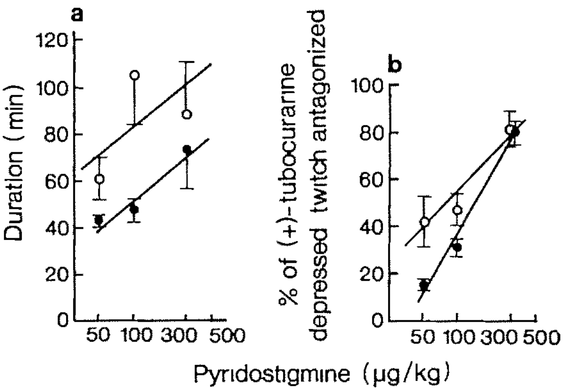

**Figure 3** Correlation between dose of pyridostigmine (plotted on a log scale) and (a) duration of antagonism of the (+)-tubocurarine depressed twitch tension and (b) the % of (+)-tubocurarine depressed twitch antagonized. Duration was defined as time from pyridostigmine administration to 50% return to the (+)-tubocurarine depressed twitch tension. Ligation of renal pedicle significantly increased the duration of pyridostigmine action. (●) Control; (O) ligated renal pedicles; each symbol represents the mean; vertical lines show s.e. mean. The number of cats studied at each dose can be obtained from Figure 4.

longer duration of action in cats with ligation than those without ligation ($P<0.10$ and $P<0.01$ respectively, Figure 3). Although the duration of action of 300 μg/kg of pyridostigmine was longer in cats with ligation these differences were not significant (Figure 3).

A 100% depression of twitch tension followed the intravenous administration of Tc, 1.0 mg/kg in four cats. Thirty minutes later, at which time the twitch was still depressed 100%, administration of neostigmine, 40 μg/kg intravenously resulted in a mean 84% antagonism of the Tc blockade. In the next 6 h twitch tension did not decrease. In fact it gradually continued to increase to the control (pre Tc) tension.

## Discussion

Our results indicate that ligation of renal pedicles prolongs the effect of both neostigmine and pyridostigmine. Yet Burdfield & Calvey (1973) found the plasma half-life of neostigmine was not prolonged by renal pedicle ligation in rats. The difference from our results may be explained by the difference in the measured variable (twitch tension and antagonism of Tc versus drug concentration in plasma). In addition to a difference in species, the problems of equating intensity of pharmacological action with plasma concentrations of drugs are well known (Koch-Weser, 1972). The importance of renal excretion to the elimination of both neostigmine and pyridostigmine is suggested by the fact that 40–60% of both neostigmine and pyridostigmine are excreted in the urine (Nowell, Scott & Wilson, 1962; Roberts, Thomas & Wilson, 1965; Birtley, Roberts, Thomas & Wilson 1966). Our results of prolonged duration of action of both drugs therefore are not surprising.

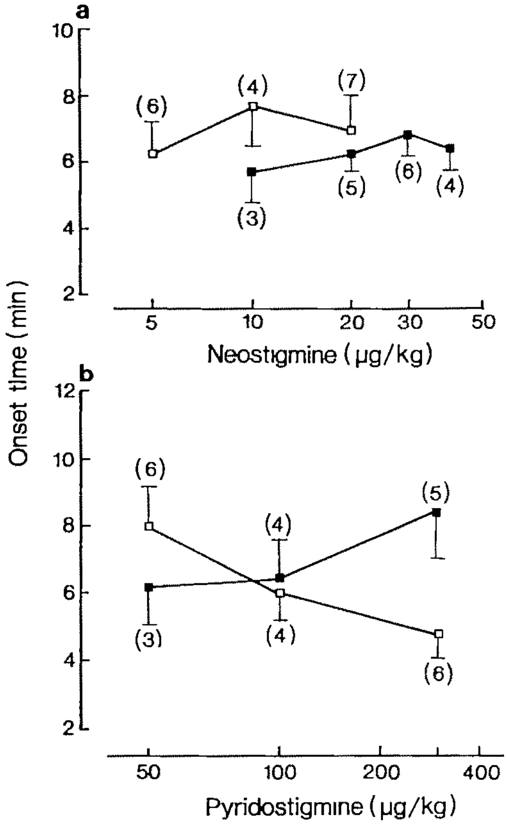

**Figure 4** Correlation between (a) dose of neostigmine or (b) pyridostigmine and time to peak antagonism. (■) Control; (□) ligated renal pedicles; each symbol represents the mean; vertical lines show s.e. mean. The numbers in parentheses represent the number of cats studied at each dose.

We believe the reduced infusion rate of Tc required for 90% depression of twitch tension and prolonged duration of neostigmine and pyridostigmine action during ligation of renal pedicles are entirely due to absence of renal function. That arterial blood pressure did not change by ligation of renal pedicles suggests that significant amounts of vasoactive substances were not released. Resting twitch tension did not change when the pedicles were ligated which suggests neuromuscular transmission was not altered. Although anaesthetic levels may have been different in the two groups of cats, chloralose has little or no effect at the cat neuromuscular junction (Hoekman, Dretchen & Standoert, 1974).

The duration of action of neuromuscular blocking drugs, Tc and pancuronium, and their antagonists, neostigmine and pyridostigmine, probably are prolonged to a similar degree by the absence of renal function (Gibaldi *et al.*, 1972; Miller, Stevens & Way, 1973; McLeod, Watson & Rawlins, 1976). This conclusion should be guarded since the duration of action of these drugs has not been studied well in the cat or man in the absence of renal function. If true, however, these results suggest that blockade from Tc should not outlast inhibition of acetylcholinesterase by neostigmine. We further tested this suggestion by administering a dose of Tc 3.5 times that required to depress twitch tension by 100% in cats with ligated renal pedicles. Subsequent administration of neostigmine resulted in a prompt and lasting antagonism of Tc. However, studies in which the pharmacokinetics or sensitivity of the neuromuscular junction to these drugs is changed by altered physiological states are required before one can assume that the blockade from TC will not outlast inhibition of acetylcholinesterase by neostigmine.

In spite of our results to the contrary, clinical reports suggest that a neuromuscular blockade may reappear when the presence Tc is prolonged by absence of renal function (Miller & Cullen, 1976; Gibaldi *et al.*, 1972) which suggests that renal excretion is more important to the termination of Tc than neostigmine action. These reports may be explained by concomitant administration of diuretics (Miller, Sohn & Matteo, 1976) or antibiotics (Pittinger & Adamson, 1972) both of which commonly are given to patients with renal failure and may augment a Tc neuromuscular blockade. Alternatively, perhaps the doses of Tc were too large and doses of neostigmine too small. These questions cannot be answered until Tc, neostigmine, and pyridostigmine concentrations and/or effects are measured in patients with and without renal failure. In any event, our results in cats indicate that the durations of neostigmine and pyridostigmine antagonism of a Tc blockade are both prolonged by renal pedicle ligation. These results suggest that the blockade from Tc should not outlast inhibition of acetylcholinesterase by neostigmine in patients with absence of renal function.

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# EFFECTS OF $\Delta^9$-TETRAHYDROCANNABINOL ON THE RATES OF OXYGEN CONSUMPTION OF MICE

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**1** Experiments with untreated mice confirmed that at ambient temperatures below 30°C, the oxygen consumption rate of mice normally kept at about 23°C varies inversely with ambient temperature.

**2** At given ambient temperatures in the range 20 to 31°C the oxygen consumption rate was 32 to 43% greater for restrained than for unrestrained mice.

**3** Hypothermia induced in restrained mice by $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) (1.0 to 4.0 mg/kg i.v.) was accompanied by marked falls in the rate of oxygen consumption. The size of these falls parallelled the degree of hypothermia and increased both with increases in dose and with decreases in the ambient temperature. The oxygen consumption rates of unrestrained mice were also lowered by hypothermic doses (10 to 40 mg/kg i.p.) of $\Delta^9$-THC.

**4** The maximum falls in oxygen consumption rate occurred at earlier times after drug administration than the maximum falls in rectal temperature.

**5** At none of the ambient temperatures studied did the oxygen consumption rates of $\Delta^9$-THC-treated mice fall significantly below the basal levels ($59 \pm 3$ ml 25 $g^{-1}$ $h^{-1}$) of unrestrained, resting mice at 30°C.

**6** The hypothesis that reduced rates of heat production contribute significantly towards the hypothermia induced by $\Delta^9$-THC in our experiments is discussed. The possibility that biological processes responsible for increased heat production in response to cold are more sensitive to $\Delta^9$-THC than those processes governing basal rates of heat production at thermally neutral environmental temperature is also raised.

## Introduction

Cannabis and its psychically active constituent $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) have been shown by several workers (see Paton & Pertwee, 1973) to affect the body temperature of a wide range of species. However, the mechanisms underlying this effect have yet to be elucidated. As a first step in the search for these mechanisms it was decided to investigate the effect of hypothermic doses of $\Delta^9$-THC on the rate of heat production by mice. Changes in rates of heat production were detected indirectly by the measurement of rates of oxygen consumption.

## Methods

Oxygen consumption rates of mice were measured by the use of a newly designed apparatus. The apparatus consisted of a closed system in which air enriched with oxygen was repeatedly circulated (500 ml/min) through an animal chamber, a container of silica gel, an oxygen analyser (Beckman OM-11), a carbon dioxide analyser (Beckman LB-2), a pump, a flowmeter and finally a container of soda lime. The animal chamber contained a single mouse. To maintain the gas pressure within the system constant, carbon dioxide generated by the mouse and absorbed by the soda lime was replaced by nitrogen. The nitrogen was stored in a spirometer and entered the system on demand through a one-way water valve. The temperature of the system could be held at any value in the range 9 to 40°C. In the experiments in which unrestrained mice were studied the total volume of the system was 505 ml (animal chamber 250 ml). In the remaining experiments the total volume was 905 ml (animal chamber 740 ml). The gas analysers provided a continuous measure of the partial pressures of oxygen and carbon dioxide in the system. At the start of each experiment, the system contained approximately 60 kPa oxygen and 40 kPa nitrogen. At no time was the partial pressure of oxygen allowed to fall below 21 kPa. The partial pressure of carbon dioxide in the system was maintained below 1.0 kPa.

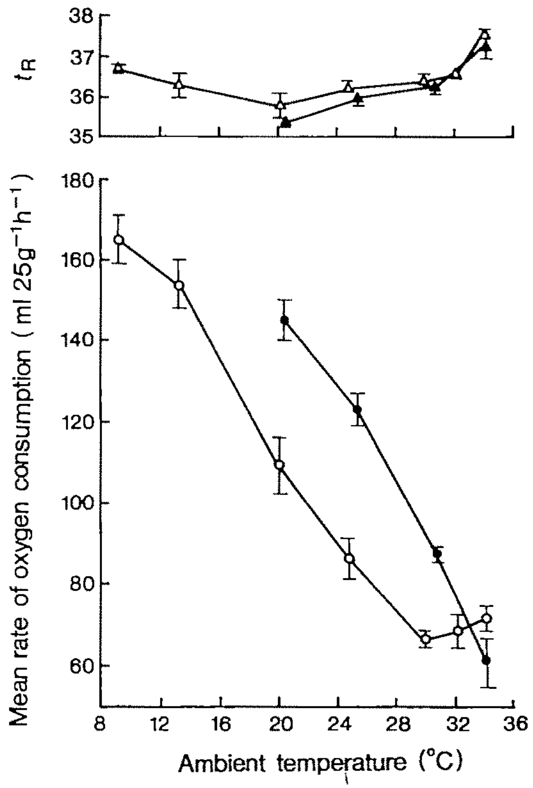

**Figure 1** The effect of ambient temperature on the mean rectal temperature ($t_R$) and oxygen consumption rates of unrestrained (open symbols) and restrained (closed symbols) groups of 6 to 9 mice. Vertical lines show s.e.

During each experiment, analyser readings were recorded at intervals of 2 to 10 minutes. This procedure allowed the mean rate at which oxygen was being consumed in each of these intervals to be calculated. Oxygen consumption rate ($VO_2$) was expressed as volume consumed per unit body weight per unit time (ml 25 g$^{-1}$ h$^{-1}$) after adjustment for s.t.p.

Adult male mice (LACA strain) weighing 28–35 g were used. The mice were maintained on a circadian cycle of 12 h light (09 h 00 min to 21 h 00 min) and 12 h darkness and received food and water *ad libitum*. Experiments were carried out both in the morning and in the afternoon. Replicates of each experimental treatment were made at both these times. The mice were injected either intraperitoneally before entry into the animal chamber or intravenously 20 to 40 min after they had been sealed in the chamber. Mice were kept in the chamber for periods of 1 to 2.5 hours. Intravenous injections were made through cannulae which had been inserted into the lateral tail veins of mice held in a restraining apparatus (Pertwee, 1970, 1974). Use of the restraining apparatus also allowed the thermistors that were used to measure body temperature to remain in position throughout an experiment. Both rectal and paw temperatures were monitored. The former was measured with a thermistor probe (Y.S.I. 402) inserted 3 cm into the rectum. Paw temperature was measured by attaching a thermistor with adhesive tape to the plantar surface of a hind paw. Measurement of paw temperature can provide an index of change in peripheral vasomotor tone (Pertwee, 1970), changes in which reflect changes in heat loss. Rectal, paw and ambient temperatures were recorded by the use of a modification of the method described by Pertwee (1970). Each of three thermistors formed in turn one arm of a Wheatstone bridge circuit. A single channel pen recorder was connected across this circuit. By the use of an electric motor and a set of microswitches (Sealectro Ltd.) the readings of the three thermistors were recorded in sequence. The readings from any one of the thermistors was registered once every minute.

For injection, $\Delta^9$-THC was mixed with two parts of Tween 80 by weight and then dispersed in 0.9% w/v NaCl solution (saline). Control injections contained doses of Tween 80 equal to or greater than those used in the corresponding drug injections. In all experiments, the volume injected was either 0.25 ml/25 g (i.p.) or 0.20 ml/25 g (i.v.).

Differences between the means of experimental data were evaluated by Student's *t* test ($P >$ or $< 0.05$) and limits of error have been expressed as standard errors. In cases where large differences in variance ruled out the use of Student's *t* test, Cochran's approximation to the Behrens-Fisher test described by Snedecor & Cochran (1973) was used.

## Results

### *Oxygen consumption rates of untreated mice*

Figure 1 shows how the oxygen consumption rates ($VO_2$) and rectal temperatures of mice which had not received any drug treatment varied with ambient temperature. The $VO_2$ of unrestrained mice decreased progressively with increase in ambient temperature over the range 9 to 30°C. At 30°C, the $VO_2$ was minimal and was not significantly different from values observed at 32 or 34°C. The $VO_2$ was also found to be significantly affected by the degree of motor activity of the mice. At 30°C, the $VO_2$ during periods of activity was 85 ± 6 ml 25 g$^{-1}$ h$^{-1}$ whereas the value of $VO_2$ during periods of rest was 59 ± 3 ml 25 g$^{-1}$ h$^{-1}$. The mean $VO_2$ for the whole of the experimental period at 30°C was 66 ± 2 ml 25 g$^{-1}$ h$^{-1}$.

When mice were secured in a restraining apparatus, there was an approximately parallel shift to the right in the ambient temperature—oxygen consumption rate curve (Figure 1), so that in the temperature range 21

**Table 1** Effects of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC, i.p.) on the oxygen consumption rates and rectal temperatures of unrestrained mice at 13°C

| *Drug* | *Dose* (*mg/kg*) | n | *Minimum rate of oxygen consumption* (*ml 25 $g^{-1}$ $h^{-1}$ ± s.e.*) | $T_1$ (*min*) | n | *Rectal temperature* *Initial* (°$C_R$ ± *s.e.*) | *Rectal temperature* *Minimum* (°$C_R$ ± *s.e.*) | $T_2$ (*min*) | $P_1$ | $P_2$ | $P_{0-2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $\Delta^9$-THC | 5 | – | – | – | 8 | 37.2±0.2 | 36.1±0.3 | 30 | – | <0.05* | <0.05 |
| Tween 80 | 80 | – | – | – | 9 | 37.4±0.2 | 36.9±0.1 | | | | <0.001 |
| $\Delta^9$-THC | 10 | 5 | 129±5 | 15 | 8 | 37.7±0.2 | 35.2±0.3 | 30 | <0.05* | <0.001 | <0.01 |
| Tween 80 | 80 | 5 | 183±19 | – | 9 | 37.4±0.2 | 36.9±0.1 | | | | <0.001 |
| $\Delta^9$-THC | 20 | 6 | 120± 8 | 30 | 8 | 37.5±0.2 | 33.0±0.6 | 30 | <0.05 | <0.001* | <0.001 |
| Tween 80 | 80 | 5 | 160±13 | – | 9 | 37.4±0.2 | 36.9±0.1 | | | | <0.001 |
| $\Delta^9$-THC | 40 | 6 | 103±6 | 20 | 8 | 37.4±0.1 | 31.4±0.4 | 30 | <0.05 | <0.001 | <0.001 |
| Tween 80 | 80 | 5 | 173±17 | – | 9 | 37.4±0.2 | 36.9±0.1 | | | | <0.001 |

Measurement of rectal temperature $t_R$ and of oxygen consumption rate ($V_{O_2}$) were made on different mice. The earliest times after injection of $\Delta^9$-THC at which maximum falls in mean $V_{O_2}$ or $t_R$ were observed are listed under $T_1$ and $T_2$ respectively Digits listed under $P_{0-2}$ are $P$ values (paired $t$ test) for differences between initial (preinjection) and minimum values of $t_R$. $P$ values (unpaired $t$ test) for differences between $\Delta^9$-THC and Tween treatments are listed under $P_1$ ($V_{O_2}$ values at $T_1$) and $P_2$ ($t_R$ values at $T_2$). The asterisk denotes use of Cochran's approximation to the Behrens Fisher test. Digits listed under $n$ refer to the number of mice used for each treatment

**Table 2** Effects of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC, I v ) on the rectal temperatures of restrained mice at an ambient temperature of 22°C

| *Drug* | *Dose* (*mg/kg*) | n | *Mean rectal temperature* *Initial* (*°C ± s.e.*) | *at* $T_1$ | *at* $T_2$ | $T_1$ (*min*) | $T_2$ | $P_1$ | $P_2$ | $P_{0-1}$ | $P_{0-2}$ | $P_{1-2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $\Delta^9$-THC | 1.0 | 5 | 36.1 ± 0.3 | 35.5 ± 0.3 | 35 0 ± 0 2 | 6 | 18 | NS | <0.05 | <0 001 | <0.01 | <0 05 |
| Tween 80 | 4.0 | 6 | 36 1 ± 0 3 | 35.9 ± 0.3 | 35.9 ± 0.3 | | | | | <0 01 | NS | NS |
| $\Delta^9$-THC | 2.0 | 6 | 36 2 ± 0.3 | 34.3 ± 0.2 | 33.1 ± 0.2 | 10 | 28 | <0 01 | <0 001 | <0.01 | <0.001 | <0.01 |
| Tween 80 | 4.0 | 6 | 36.1 ± 0.3 | 36.0 ± 0.3 | 35.9 ± 0.3 | | | | | NS | NS | NS |
| $\Delta^9$-THC | 4.0 | 5 | 36.0 ± 0.4 | 32.6 ± 0.3 | 30.8 ± 0.6 | 14 | 36 | <0.001 | <0 001 | <0.01 | <0.01 | <0.01 |
| Tween 80 | 8.0 | 6 | 36.7 ± 0.1 | 36.6 ± 0.1 | 36.7 ± 0.1 | | | | | NS | NS | NS |

The earliest times (minutes) after injection of $\Delta^9$-THC at which maximum falls in mean oxygen consumption rate or in mean rectal temperature were observed are listed in the right hand columns under $T_1$ and $T_2$ respectively. Digits listed under $P_1$ and $P_2$ are $P$ values (unpaired $t$ test) for differences between $\Delta^9$-THC and Tween treatments at the times $T_1$ and $T_2$ respectively. $P$ values (paired $t$ test) for differences between preinjection values and values obtained at the times $T_1$ and $T_2$ are listed under $P_{0-1}$ and $P_{0-2}$ respectively. $P$ values (paired $t$ test) for differences between values obtained at $T_1$ and $T_2$ are listed under $P_{1-2}$. The abbreviation NS indicates a $P$ value >0 05 Digits listed under $n$ refer to the number of mice used for each experimental treatment.

**Table 3** Effects of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC, i.v.) on the oxygen consumption rates of restrained mice at an ambient temperature of 22°C

| *Drug* | *Dose* (*mg/kg*) | n | *Mean oxygen consumption rate* *Initial* (*ml 25 $g^{-1}$ $h^{-1}$ ± s.e.*) | *at* $T_1$ | *at* $T_2$ | $T_1$ (*min*) | $T_2$ | $P_1$ | $P_2$ | $P_{0-1}$ | $P_{0-2}$ | $P_{1-2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $\Delta^9$-THC | 1.0 | 5 | 140 ± 1 | 115 ± 7 | 126 ± 4 | 6 | 18 | <0.01 | <0 05 | <0.05 | <0.05 | NS |
| Tween 80 | 4.0 | 6 | 150 ± 4 | 166 ± 10 | 146 ± 7 | | | | | NS | NS | <0 05 |
| $\Delta^9$-THC | 2.0 | 6 | 152 ± 4 | 84 ± 7 | 115 ± 6 | 10 | 28 | <0 01 | <0 001 | <0.001 | <0.05 | <0.05 |
| Tween 80 | 4.0 | 6 | 150 ± 4 | 136 ± 13 | 153 ± 6 | | | | | NS | NS | NS |
| $\Delta^9$-THC | 4.0 | 5 | 145 ± 6 | 58 ± 6 | 89 ± 9 | 14 | 36 | <0.001 | <0 01 | <0.001 | <0.01 | <0.01 |
| Tween 80 | 8.0 | 6 | 148 ± 3 | 148 ± 10 | 148 ± 5 | | | | | NS | NS | NS |

The values of mean oxygen consumption rate in this table were obtained in the same experiments as the values of mean rectal temperature given in Table 2 See also footnote to Table 2.

to 31°C, the $V_{O_2}$ at any given ambient temperature was 32 to 43% greater for restrained mice than for unrestrained animals. At 34°C, the $V_{O_2}$ of restrained mice was $60 \pm 6$ ml 25 $g^{-1}$ $h^{-1}$, a value which was not significantly different from the minimum $V_{O_2}$ values of unrestrained mice.

### *Effects of $\Delta^9$-THC on the oxygen consumption rates of mice*

The first experiments were carried out on unrestrained mice at 13°C and the results are summarized in Table 1. Administration of $\Delta^9$-THC (10 to 40 mg/kg i.p.) produced significant, reversible falls in $V_{O_2}$. Experiments with different groups of mice showed that the above drug treatments also induced a significant degree of hypothermia. Small but statistically significant falls in rectal temperature were also observed in mice that received only Tween 80. However, at all the doses of $\Delta^9$-THC that were studied, the hypothermia following the injection of the drug was significantly greater than that following the injection of Tween 80. Maximum falls in $V_{O_2}$ ranged from 29 to 40% of $V_{O_2}$ values of a Tween-treated control group. At the same doses of $\Delta^9$-THC, maximum falls in rectal temperature ranged from 2.5°C to 6.0°C.

Subsequent experiments were carried out with mice secured to a restraining apparatus and fitted with an intravenous cannula and with temperature probes. This procedure, unlike the one used in the preliminary experiments, allowed measurements of $V_{O_2}$ to be made (1) simultaneously with the measurement of body temperature and (2) before, during and immediately after drug administration. The experiments were carried out at 22°C. At this ambient temperature, $\Delta^9$-THC (1.0 to 4.0 mg/kg i.v.) produced significant falls both in $V_{O_2}$ and in rectal temperature. The results are summarized in Tables 2 and 3. A dose of 1.0 mg/kg produced a peak fall in $V_{O_2}$ of $17 \pm 5$% of the mean pre-injection value whereas doses of 2.0 and 4.0 mg/kg produced peak falls of $44 \pm 5$% and $60 \pm 5$% respectively. The degree of hypothermia produced by $\Delta^9$-THC also increased progressively with dose. Tween 80 produced a slight, transient fall in rectal temperature in one group of animals (Table 2). In all the other experiments at 22°C, administration of Tween 80 alone produced no significant decreases either in $V_{O_2}$ or in rectal temperature.

The falls in $V_{O_2}$ produced by $\Delta^9$-THC significantly affected the volume of oxygen consumed by mice during the onset of hypothermia. A dose of 1.0 mg/kg produced a fall in the volume of oxygen consumed over the period in which rectal temperature fell to a minimum value that was 21% of the volume consumed in the same interval of time (18 min) by a Tween-treated group of mice. Doses of 2.0 and 4.0 mg/kg produced falls in the volume of oxygen consumed during the onset of hypothermia which were respectively 32% and 49% of control values.

The time taken after intravenous administration of $\Delta^9$-THC for $V_{O_2}$ and rectal temperature to reach minimum values was not constant and increased progressively with dose. At each dose, the maximum fall in $V_{O_2}$ occurred at an earlier time than the maximum fall in rectal temperature. Falls in rectal temperature were maximal 18 to 36 min after injection whereas maximal falls in $V_{O_2}$ took place in 6 to 14 minutes. At the latter times rectal temperatures, although already significantly below pre-injection values, were still significantly above the minimum values observed 18 to 36 min after injection. Furthermore, in groups of mice that had received $\Delta^9$-THC at doses of 2.0 or 4.0 mg/kg, a significant recovery of $V_{O_2}$ was observed at the times of peak hypothermia.

### *Effects of ambient temperature on changes in oxygen consumption rate induced in restrained mice by $\Delta^9$-THC at a dose of 2.0 mg/kg (i.v.)*

Tables 4 and 5 show how changes in ambient temperature in the range 22 to 34°C influenced the effect of $\Delta^9$-THC on $V_{O_2}$ and rectal temperature. Significant, reversible falls in both $V_{O_2}$ and rectal temperature were induced by the drug throughout this range. In contrast, at none of the ambient temperatures studied was any significant reduction in $V_{O_2}$ produced by Tween 80. Figures 2 and 3 show the time courses of the effects of $\Delta^9$-THC and of Tween 80 both on the rectal temperature and on the $V_{O_2}$ of groups of mice at an ambient temperature of 27°C.

In the range 22 to 34°C, the size of the fall in $V_{O_2}$ that followed injection of $\Delta^9$-THC decreased progressively with increase in ambient temperature. At 22°C the maximum fall in $V_{O_2}$ was 68 ml 25 $g^{-1}$ $h^{-1}$ whereas at 34°C the maximum fall was only 23 ml 25 $g^{-1}$ $h^{-1}$. Minimum values of $V_{O_2}$ following $\Delta^9$-THC ranged from $84 \pm 7$ ml 25 $g^{-1}$ $h^{-1}$ at 22°C to $52 \pm 5$ ml 25 $g^{-1}$ $h^{-1}$ at 30°C. The minimum values of $V_{O_2}$ at 32 and 34°C were not significantly different from the value at 30°C. The degree of hypothermia produced by $\Delta^9$-THC also varied inversely with ambient temperature. Minimum values of rectal temperature ranged from $33.1 \pm 0.2$°C at 22°C to $37.3 \pm 0.1$°C at 34°C.

As in the experiments carried out at 22°C, the maximum fall in $V_{O_2}$ following the injection of $\Delta^9$-THC at each of the higher ambient temperatures studied, occurred at an earlier time than did the maximum fall in rectal temperature. Furthermore, at the times at which the maximum fall in $V_{O_2}$ occurred, the degree of hypothermia although already significant was far from maximal. In addition, at 27 and 32°C, a significant recovery of $V_{O_2}$ was observed at the times of peak hypothermia.

**Table 4** The influence of ambient temperature on the effects of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC, 2.0 mg/kg i.v.) and Tween 80 (4.0 mg/kg i.v.) on the rectal temperature of restrained mice

| *Drug* | *Ambient temperature (°C)* | n | *Mean rectal temperature* *Initial* | *at $T_1$* (°C ± s.e.) | *at $T_2$* | $T_1$ (min) | $T_2$ (min) | $P_1$ | $P_2$ | $P_{0-1}$ | $P_{0-2}$ | $P_{1-2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $\Delta^9$-THC | 22 | 6 | 36.2±0.3 | 34.3±0.2 | 33.1±0.2 | 10 | 28 | <0.01 | <0.001 | <0.01 | <0.001 | <0.001 |
| Tween 80 | | 6 | 36.1±0.3 | 36.0±0.3 | 35.9±0.3 | | | | | NS | NS | NS |
| $\Delta^9$-THC | 27 | 6 | 36.9±0.1 | 36.2±0.2 | 34.9±0.3 | 6 | 18 | NS | <0.05 | <0.01 | <0.001 | <0.001 |
| Tween 80 | | 5 | 36.2±0.2 | 36.1±0.2 | 36.1±0.3 | | | | | NS | NS | NS |
| $\Delta^9$-THC | 30 | 6 | 37.3±0.2 | 36.0±0.1 | 35.9±0.1 | 12 | 14 | <0.001 | <0.001 | <0.001 | <0.001 | <0.05 |
| Tween 80 | | 6 | 37.2±0.2 | 37.4±0.2 | 37.4±0.2 | | | | | <0.05 | NS | NS |
| $\Delta^9$-THC | 32 | 6 | 37.5±0.2 | 36.8±0.2 | 36.6±0.2 | 16 | 20 | <0.05 | <0.01 | <0.01 | <0.01 | <0.05 |
| Tween 80 | | 6 | 37.3±0.2 | 37.4±0.2 | 37.5±0.2 | | | | | NS | NS | NS |
| $\Delta^9$-THC | 34 | 6 | 38.1±0.1 | 37.6±0.1 | 37.3±0.1 | 14 | 24 | NS | <0.001 | <0.01 | <0.01 | <0.05 |
| Tween 80 | | 6 | 37.9±0.2 | 37.9±0.2 | 38.0±0.1 | | | | | NS | NS | <0.05 |

For details see footnote to Table 2.

**Table 5** The influence of ambient temperature on the effects of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC, 2.0 mg/kg i.v.) and Tween 80 (4.0 mg/kg i.v.) on the oxygen consumption rates of restrained mice

| Drug | *Temperature (°C)* | n | *Mean oxygen consumption rate* *Initial* | *at $T_1$* (ml 25 $g^{-1}$ $h^{-1}$) | *at $T_2$* | $T_1$ (min) | $T_2$ (min) | $P_1$ | $P_2$ | $P_{0-1}$ | $P_{0-2}$ | $P_{1-2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $\Delta^9$-THC | 22 | 6 | 152±4 | 84±7 | 115±6 | 10 | 28 | <0.01 | <0.001 | <0.001 | <0.05 | <0.05 |
| Tween 80 | | 6 | 150±4 | 136±13 | 153±6 | | | | | NS | NS | NS |
| $\Delta^9$-THC | 27 | 6 | 120±3 | 61±4 | 90±5 | 6 | 18 | <0.001 | NS | <0.001 | <0.001 | <0.01 |
| Tween 80 | | 5 | 107±5 | 115±7 | 105±9 | | | | | NS | NS | NS |
| $\Delta^9$-THC | 30 | 6 | 95±4 | 52±5 | 57±7 | 12 | 14 | <0.001 | <0.01 | <0.01 | <0.05 | NS |
| Tween 80 | | 6 | 86±4 | 85±4 | 94±5 | | | | | NS | NS | NS |
| $\Delta^9$-THC | 32 | 6 | 84±4 | 50±4 | 79±9 | 16 | 20 | <0.01 | NS | <0.001 | NS | <0.01 |
| Tween 80 | | 6 | 74±3 | 75±4 | 76±6 | | | | | NS | NS | NS |
| $\Delta^9$-THC | 34 | 6 | 71±3 | 48±5 | 57±7 | 14 | 24 | NS | NS | <0.01 | NS | NS |
| Tween 80 | | 6 | 71±3 | 54±7 | 65±3 | | | | | NS | NS | NS |

The values of oxygen consumption rate in this table were obtained in the same experiments as the values of rectal temperature given in Table 4. See also footnote to Table 2.

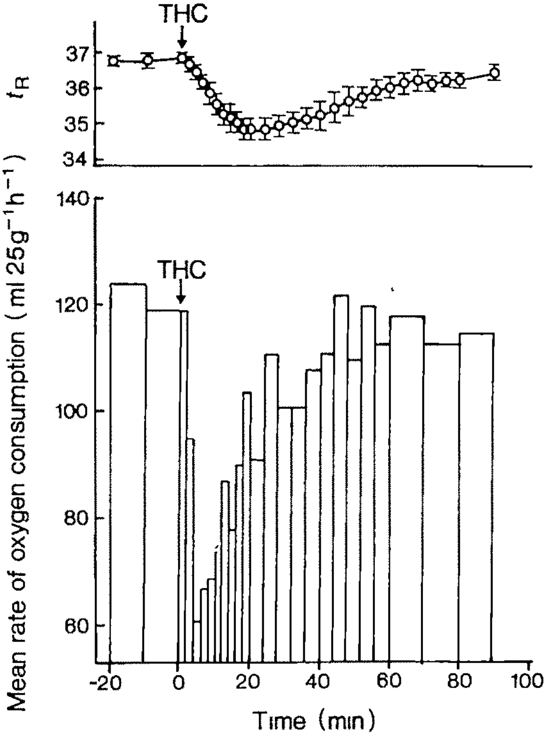

**Figure 2** The effect of Δ$^9$-tetrahydrocannabinol (THC, 2.0 mg/kg i.v) on the mean rectal temperatures ($t_R$) and oxygen consumption rates of a group of 6 mice kept separately at an ambient temperature of 27°C. The arrow denotes the time of injection. Vertical lines show s.e.

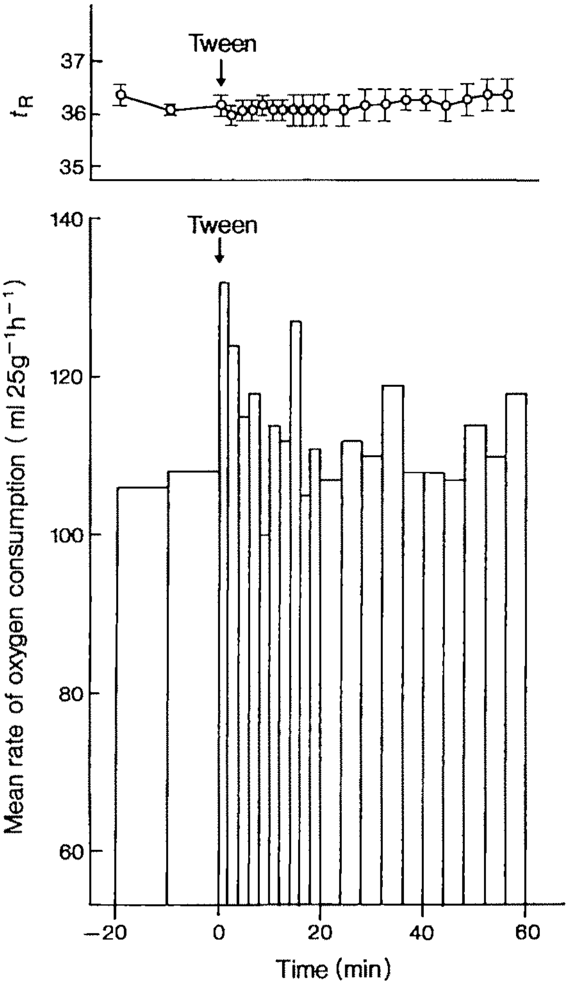

**Figure 3** The effect of Tween 80 (4.0 mg/kg i.v.) on the mean rectal temperatures ($t_R$) and oxygen consumption rates of a group of 5 mice kept separately at an ambient temperature of 27°C. The arrow denotes the time of injection. Vertical lines show s.e.

### *Effects of Δ$^9$-THC on the paw temperatures of restrained mice*

At ambient temperatures of 27, 30 and 34°C the hypothermia induced by Δ$^9$-THC at a dose of 2.0 mg/kg (i.v.) was accompanied by significant falls in paw temperature. At each of these ambient temperatures the maximum fall in paw temperature occurred at a later time than the maximum fall in rectal temperature (see Tables 4 and 6). In contrast, at temperatures of 22 and 32°C, Δ$^9$-THC had no significant effect on paw temperature. Administration of Tween 80 alone produced no significant falls in paw temperature at any of the ambient temperatures that were used.

## Discussion

The results confirm earlier observations that Δ$^9$-THC can lower the body temperature of mice and that the degree of hypothermia produced is dependent not only on dose level but also on the ambient temperature (Haavik & Hardman, 1973). The experiments also showed that hypothermia induced in mice by Δ$^9$-THC is accompanied by marked falls in the rate of oxygen consumption. The results therefore provide indirect evidence for the concept that hypothermia induced in mice by Δ$^9$-THC is associated with reduced rates of heat production. The results also support the hypothesis that there is a cause and effect relationship between reduced oxygen consumption and hypothermia, the latter resulting from an effect of Δ$^9$-THC on oxygen utilizing processes of the body. Firstly, the effect of Δ$^9$-THC on oxygen consumption correlates

**Table 6** Effects of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC, 2.0 mg/kg i.v.) and Tween 80 (4.0 mg/kg i.v.) on the paw temperature of restrained mice

| *Drug* | *Ambient temperature (°C)* | n | *Mean paw temperature* *Initial* (°C ± s.e.) | *Mean paw temperature* *at $T_{max}$* (°C ± s.e.) | $T_{max}$ *(min)* | P |
|---|---|---|---|---|---|---|
| $\Delta^9$-THC | 22 | 6 | 26.1 ± 0.5 | 25.2 ± 0.4 | 28 | NS |
| Tween 80 | | 6 | 27.9 ± 0.6 | 27.3 ± 0.5 | | NS |
| $\Delta^9$-THC | 27 | 6 | 29.1 ± 0.5 | 28.5 ± 0.4 | 24 | $<0.05$ |
| Tween 80 | | 5 | 28.7 ± 0.5 | 28.9 ± 0.6 | | NS |
| $\Delta^9$-THC | 30 | 6 | 33.5 ± 0.5 | 32.0 ± 0.3 | 28 | $<0.05$ |
| Tween 80 | | 6 | 32.6 ± 0.5 | 32.5 ± 0.6 | | NS |
| $\Delta^9$-THC | 32 | 6 | 34.3 ± 0.6 | 33.1 ± 0.1 | 32 | NS |
| Tween 80 | | 6 | 34.6 ± 0.5 | 35.1 ± 0.6 | | $<0.05$ |
| $\Delta^9$-THC | 34 | 6 | 36.6 ± 0.5 | 34.5 ± 0.2 | 40 | $<0.05$ |
| Tween 80 | | 6 | 35.6 ± 0.6 | 36.3 ± 0.5 | | NS |

The earliest times (minutes) after injection of $\Delta^9$-THC at which maximum falls in mean paw temperature were observed are listed under $T_{max}$. Digits listed under *P* denote the *P* values (paired *t* test) for differences between preinjection and postinjection values. Digits listed under *n* refer to the number of mice used for each treatment. The values of paw temperature in this table were obtained in the same experiments as the values of rectal temperature given in Table 4.

well with the hypothermia produced by the drug; both parameters increase with increase in dose of $\Delta^9$-THC and with decrease in the ambient temperature. Secondly, the falls in oxygen consumption rate produced by the drug were always found to take place earlier than the falls in body temperature. Furthermore, in many of the experiments, a significant reversal of the effect of $\Delta^9$-THC on oxygen consumption had already occurred at the time at which peak hypothermia was first observed. It should be noted however, that the latter findings may simply be a reflection of the tendency of rectal temperature to lag behind changes in the temperatures of other internal parts of the body.

Our measurements of paw temperature showed that the hypothermic doses of $\Delta^9$-THC used in our experiments produced only falls in paw temperature. There was no sign therefore that increased rates of peripheral heat loss contributed significantly towards the hypothermia induced in mice by $\Delta^9$-THC. However, this possibility cannot yet be ruled out since it is not clear whether paw temperature would necessarily become elevated as a result of peripheral vasodilatation in mice that were at the same time experiencing progressive decreases in deep body temperature. It should be noted that maximum falls in paw temperature occurred at later times than maximum falls in rectal temperature. It is possible therefore that the falls in paw temperature are caused by vasoconstriction of peripheral blood vessels and that this vasoconstriction contributes towards recovery from drug-induced hypothermia.

Experiments with untreated mice confirm earlier reports (Herrington, 1940) that at ambient temperatures below 30°C, the oxygen consumption rate of mice normally kept at about 23°C varied inversely with ambient temperature. The experiments also showed that at an ambient temperature of 30°C, rates of oxygen consumption during periods in which mice exhibited increased motor activity were 44% greater than during periods in which the animals were resting. Increases in oxygen consumption were also observed when mice were secured in a restraining apparatus. In the range 22–32°C, oxygen consumption rates at any given ambient temperature were 32 to 43% greater in immobilized mice than in unrestrained animals. Similar effects of restraint on oxygen consumption have been found to occur with both guinea-pigs (Bartlett, 1959) and rabbits (McEwen, 1975). The method of restraint used in our experiments has been shown previously (Pertwee, 1974) to be a stressful procedure as measured by its effect on plasma corticosterone concentrations in mice. It is therefore possible that the increase in oxygen consumption which takes place when the mice are immobilized may be induced by stress. In view of the increase in plasma corticosterone produced in mice by restraint it is important to note that hypothermic doses of $\Delta^9$-THC were found to lower the oxygen consumption not only of immobilized mice but also of unrestrained animals. It should also be noted that the doses of $\Delta^9$-THC found to produce hypothermia in unrestrained animals have themselves been shown in previous experiments (Pertwee, 1974) to elevate plasma corticosterone in mice. Consequently the question of whether or not the effects of $\Delta^9$-THC on oxygen consumption and rectal temperature are influenced by the degree of stress experienced by the experimental animals cannot yet be answered.

Finally, it was observed that the size of the effect of $\Delta^9$-THC on oxygen consumption rate was dependent on the rate of consumption before the drug injection was made. Both the size of the effect of $\Delta^9$-THC on oxygen consumption rate and the pre-injection values of this parameter increased with decrease in ambient temperatures. It was also observed that the oxygen consumption rates of mice treated with hypothermic doses of $\Delta^9$-THC never fell significantly below the basal levels observed in unrestrained, resting mice at 30°C. If reduced rates of heat production do indeed contribute significantly towards production of the hypothermia described in this paper, then the above findings support the hypothesis that the biological processes responsible for increased heat production in response to cold are more sensitive to $\Delta^9$-THC than are the biological processes governing the basal rates of metabolism observed at thermally neutral environmental temperatures.

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# AN INVESTIGATION OF THE SEX DIFFERENCE IN THE DIURETIC RESPONSE TO ETHACRYNIC ACID IN MICE

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1 Female mice of the Hough/Porton and Tuck/TO strains were found to be more sensitive than male mice to the diuretic effects of oral and intravenous doses of ethacrynic acid.

2 The sensitivity of Hough/Porton male mice to ethacrynic acid was increased after pretreatment with stilboestrol and the sensitivity of female Hough/Porton mice decreased after pretreatment with testosterone.

3 There were no significant sex differences in the diuretic response to frusemide, acetazolamide, aminophylline, bendrofluazide, and Su 15049A although a small, but significant, increase in the sensitivity of male Tuck/TO mice to triamterene was noted.

4 The sex difference in diuretic response to ethacrynic acid may be related to an effect of sex hormone balance on its metabolism or on the sensitivity of its renal receptor.

## Introduction

In a recent paper describing a method for evaluating diuretics in mice (Sim & Hopcroft, 1976) it was emphasized that this species responded to ethacrynic acid, an agent which is inactive in the rat (Beyer, Baer, Michaelson & Russo, 1965). In preliminary studies (unpublished) we noted that female mice were more sensitive to ethacrynic acid, an effect recently reported by Hill & Randall (1976). We decided to study this sex difference in greater detail and to examine possible sex differences in the response to other diuretics with differing modes of action including Su 15049A, a compound with a novel mode of action which has not been used clinically (Gaunt, Renzi, Howie, Gisoldi & Waldron, 1967).

## Methods

### *Measurement of diuretic effects*

The method was that described by Sim & Hopcroft (1976). Hough/Porton male (17 to 34 g) and female (15 to 32 g) mice and Tuck/TO male (15 to 25 g) and female (15 to 24 g) mice were used. In comparative experiments the weight range did not exceed 10 grams.

Mice, housed in groups of 35, were fasted overnight but allowed free access to water. On the morning of the experiment the mice were distributed randomly into groups of five and placed in small cages for 1 to 2 h before dosing. Oral doses of diuretics were given as suspensions or solutions in 30 ml/kg 0.25% cellosize in glass distilled water. Controls received the same volume of vehicle alone. Ethacrynic acid for intravenous doses was dissolved in sterile water with a minimal quantity of 1 N NaOH to pH 7.4 to 7.7; volumes of 5 ml/kg were injected via the tail vein. In intravenous studies all mice were given an oral load of 30 ml/kg 0.25% cellosize in glass-distilled water immediately before the intravenous dose. After dosing, mice were placed in a metabolism cage (Sim & Hopcroft, 1976) and urine was collected in a graduated tube for 3 hours. For comparison, all dose levels were tested on the same day and the experiment repeated at least four times.

### *Pretreatment with sex hormones*

Hough/Porton mice from the same batch were distributed randomly into two groups; one group received hormone pretreatment, the other no pretreatment. Male mice were pretreated orally four days before the test with a single dose of 250 mg/kg stilboestrol suspended in 0.25% cellosize in glass-distilled water. Female mice were given 100 mg/kg testosterone subcutaneously on each of the two days before the test. The testosterone was dissolved in arachis oil to give 20 mg/ml. These large hormone doses are in the same range as those used in mice by Westfall, Boulos, Shields & Garb (1964).

### *Toxicity determination*

Oral $LD_{50}$ values were determined in male and female Tuck/TO mice weighing 20 to 25 grams. The mice were fasted overnight but allowed free access to water. Ethacrynic acid in a suitable range of doses was administered to groups of 10 animals of each sex in a volume of 45 ml/kg 0.25% cellosize in glass-distilled water. Controls received the same volume of vehicle alone. The mice were observed for a period of seven days and the number of deaths noted. LD$_{50}$ values were calculated by a probit analysis method.

### *Drugs*

The drugs used were: acetazolamide (Diamox powder; Cyanamid of Great Britain), aminophylline B.P., bendrofluazide B.P. (Boots Company), ethacrynic acid B.P. (Merck, Sharp & Dohme), frusemide B.P. (Hoechst Pharmaceuticals), triamterene B.P. (Smith, Kline & French) and Su 15049A, the citrate of Su 15049 (2-2,6-diphenyl-4-(1-pyrrolidinyl)cyclohexyl pyridine) (Ciba-Geigy). Doses are expressed in terms of the appropriate acid or base except for Su 15049A which is expressed as the salt. Cellosize (hydroxyethyl cellulose grade QP15000) was obtained from Union Carbide.

Diethylstilboestrol and testosterone propionate were obtained from the Sigma Chemical Co. and doses are expressed in terms of the base and salt respectively.

## Results

In this study sodium ion excretion has been used as an index of diuretic activity since it is a more sensitive indicator than volume excretion (Sim & Hopcroft, 1976). Potency is related to the amount of drug required to produce a given sodium ion excretion and efficacy is related to the maximal sodium ion excretion.

Sodium ion excretion in control male and female Hough/Porton mice was approximately the same but ethacrynic acid had a greater diuretic potency in female mice than in male mice after oral or intravenous administration (Table 1).

The sex difference in response to ethacrynic acid was not confined to Hough/Porton strain mice since a difference was also observed with Tuck/TO strain mice (Table 2). In contrast there was no sex difference in the diuretic response to frusemide, a high efficacy diuretic which like ethacrynic acid has its main effect on the loop of Henle (Table 2). The effects of five other diuretics were studied in male and female Hough/Porton and Tuck/TO mice using doses which were found to be submaximal in a previous study (Sim & Hopcroft, 1976). These diuretics which have differing modes of actions are all less efficacious than either ethacrynic acid or frusemide in mice (Sim & Hopcroft, 1976). There was a small but significant sex difference in the diuretic response to triamterene in Tuck/TO strain mice, in which male mice were more sensitive than female mice, but this difference was not observed in Hough/Porton mice. There were no significant sex differences in either strain in response to the four other compounds (Table 2).

We investigated this sex difference by examining the effect of exogenous sex hormones on the diuretic response. Pretreatment of male Hough/Porton mice with stilboestrol significantly reduced control sodium excretion but significantly increased the sensitivity to ethacrynic acid (Table 3). Testosterone did not significantly alter sodium excretion in control female Hough/Porton mice but significantly reduced the sensitivity to ethacrynic acid in this sex (Table 3).

**Table 1** The diuretic effect of oral and intravenous doses of ethacrynic acid in male and female Hough/Porton mice

| | | Na$^+$ (mmol/kg) | | | |
|---|---|---|---|---|---|
| *Route of administration* | *Dose (mg/kg)* | *Male* | n | *Female* | n |
| Oral | 0 | $0.65 \pm 0.11$ | (8) | $0.52 \pm 0.06$ | (5) |
| | 1 | $0.92 \pm 0.16$ | (9) | $1.58 \pm 0.18^{**}$ | (10) |
| | 10 | $3.35 \pm 0.49$ | (10) | $6.06 \pm 0.55^{***}$ | (10) |
| | 100 | $6.35 \pm 0.79$ | (10) | $7.80 \pm 0.48$ | (10) |
| Intravenous | 0 | $0.60 \pm 0.11$ | (9) | $0.49 \pm 0.06$ | (9) |
| | 1 | $1.57 \pm 0.16$ | (9) | $2.24 \pm 0.21^{**}$ | (9) |
| | 10 | $2.96 \pm 0.18$ | (9) | $5.43 \pm 0.37^{***}$ | (9) |
| | 100 | $8.90 \pm 0.56$ | (9) | $9.51 \pm 0.53$ | (9) |

Each value is mean Na$^+$ excretion at 3 h ± s.e. for *n* groups of five mice. Ethacrynic acid was given orally in 30 ml/kg 0.25% cellosize in glass-distilled water, controls received the same volume of vehicle alone. Intravenous doses of ethacrynic acid were injected via the tail vein at 5 ml/kg immediately after an oral load of 30 ml/kg 0.25% cellosize in glass-distilled water. Urine was collected for 3 hours

$^{***}P < 0.001$; $^{**}P < 0.01$ for differences in response between sexes at the same dose level (Student's *t* test).

Oral $LD_{50}$ values in male and female Tuck/TO mice were 621 mg/kg and (95% fiducial limits 597 to 647 mg/kg) and 562 mg/kg (95% fiducial limits 522 to 605 mg/kg) respectively.

## Discussion

These results have demonstrated a significantly increased sensitivity to the diuretic effects of ethacrynic acid in female mice compared with males in two different strains. Hill & Randall (1976) using a third strain (120/OLA) also found that female mice were more sensitive to ethacrynic acid but found no sex difference in response to other diuretics. In the present study there was also a sex difference in response to triamterene in one strain (Tuck/TO) but the difference was comparatively small and it was male mice in this case that were more sensitive than females.

Hill & Randall (1976) reported that their female mice were either in dioestrus or anoestrus at the time of their experiments. We did not determine the stage of oestrus in our mice but the animals used were four to six weeks old and were approaching sexual maturity. Our results suggest that the balance between oestrogen and androgen levels is an important factor influencing the response to ethacrynic acid since large doses of stilboestrol can increase the senstivity in male mice and large doses of testosterone can decrease the sensitivity in female mice. A hormone-dependent effect in mice has also been observed for barbiturates. Westfall *et al.* (1964) observed that the sex difference in the hypnotic response of mice to pentobarbitone could be reversed by treating male mice with stilboestrol and female mice with testosterone.

Differences in oral absorption, metabolism or in the sensitivity of the renal receptor mechanism might be responsible for the sex difference. The first possibility was excluded since the sex difference occurred after oral or intravenous administration of ethacrynic acid.

The sex difference in the hypnotic effect of barbiturates in mice may be due to differences in metabolism (Vesell, 1968) and we have, therefore, given some consideration as to whether differences in metabolism could account for our results. A major excretion product after a single dose of ethacrynic acid in rats and dogs is the cysteine adduct (EA-Cyst) (Beyer *et al.*, 1965; Klaassen & Fitzgerald, 1974). Burg & Green (1973) suggested that EA-Cyst is the major active form of the drug *in vivo* since it was 100 times as potent as the parent compound as an inhibitor of chloride transport in isolated cortical loops of Henle from rabbit kidney.

**Table 2** Effects of diuretic agents on sodium excretion in male and female Hough/Porton or Tuck/TO mice

| *Compound* | *Oral dose (mg/kg)* | n | *Strain* | *$Na^+$(mmol/kg)* | |
|---|---|---|---|---|---|
| | | | | *Male* | *Female* |
| Ethacrynic acid | 10 | 6 | Hough/Porton | 2.39±0.48 | 5.71±0.43*** |
| | | 6 | Tuck/TO | 2.13±0.15 | 5.29±0.53** |
| Ethacrynic acid | 30 | 6 | Hough/Porton | 4.29±0.64 | 8.32±0.44*** |
| | | 6 | Tuck/TO | 4.05±0.44 | 6.87±0.40*** |
| Ethacrynic acid | 100 | 6 | Hough/Porton | 5.82±0.94 | 8.23±0.30* |
| | | 6 | Tuck/TO | 6.23±0.45 | 8.18±0.59* |
| Frusemide | 10 | 6 | Hough/Porton | 4.50±0.37 | 4.50±0.26 |
| | | 6 | Tuck/TO | 4.28±0.41 | 4.77±0.62 |
| Frusemide | 30 | 6 | Hough/Porton | 7.10±0.49 | 7.46±0.31 |
| | | 6 | Tuck/TO | 6.45±0.35 | 6.74±0.36 |
| Frusemide | 100 | 6 | Hough/Porton | 8.23±0.38 | 8.74±0.32 |
| | | 6 | Tuck/TO | 7.69±0.55 | 7.77±0.34 |
| Bendrofluazide | 10 | 6 | Hough/Porton | 1.95±0.44 | 2.78±0.35 |
| | | 4 | Tuck/TO | 2.03±0.50 | 2.09±0.11 |
| Acetazolamide | 10 | 6 | Hough/Porton | 1.38±0.34 | 1.59±0.32 |
| | | 4 | Tuck/TO | 2.36±0.17 | 2.43±0.22 |
| Triamterene | 10 | 6 | Hough/Porton | 1.16±0.14 | 1.18±0.21 |
| | | 4 | Tuck/TO | 2.85±0.46 | 1.29±0.30* |
| Aminophylline | 30 | 6 | Hough/Porton | 1.72±0.27 | 1.70±0.12 |
| | | 4 | Tuck/TO | 2.49±0.43 | 2.44±0.20 |
| Su 15049A | 10 | 6 | Hough/Porton | 2.12±0.59 | 1.86±0.36 |
| | | 4 | Tuck/TO | 1.58±0.33 | 2.10±0.20 |

Each value is mean $Na^+$ excretion at 3 h ± s.e for *n* groups of five mice. The diuretic agents were given orally in a volume of 30 ml/kg 0.25% cellosize in glass-distilled water. Urine was collected for 3 hours.
*** $P=<0.001$; ** $P=<0.01$; * $P=<0.05$ for differences in response between the sexes at the same dose level (Student's *t* test).

**Table 3** Effect of pretreatment with sex hormones on the diuretic response of male and female Hough/Porton mice to ethacrynic acid

| | *Ethacrynic acid oral dose (mg/kg)* | n | *Na⁺ (mmol/kg)* | |
|---|---|---|---|---|
| | | | *Untreated* | *Pretreated* |
| (a) Male mice pretreated with stilboestrol | 0 | (9) | 0.54±0.12 | 0.20±0.04* |
| | 10 | (9) | 2.13±0.15 | 3.08±0.20*** |
| | 30 | (9) | 3.46±0.14 | 6.16±0.28*** |
| | 100 | (9) | 5.00±0.25 | 8.69±0.70*** |
| (b) Female mice pretreated with testosterone | 0 | (3) | 0.77±0.13 | 0.58±0.17 |
| | 10 | (5) | 6.98±0.41 | 2.77±0.22*** |
| | 30 | (5) | 8.36±0.55 | 4.52±0.24*** |
| | 100 | (5) | 8.97±0.53 | 6.21±0.18*** |

Each value is mean ± s.e. for *n* groups of five mice Stilboestrol was given as a single oral dose 250 mg/kg four days before the test, testosterone at 100 mg/kg subcutaneously on each of the two days before the test Ethacrynic acid was given in 30 ml/kg 0.25% cellosize in glass distilled water.

*** $P=<0.001$; **$P=<0.01$; *$P=<0.05$ for differences in response between untreated and pretreated mice at the same dose level (Student's *t* test)

Burg (1976) has proposed that the formation of EA-Cyst should enhance the diuretic effect but reduce the toxicity of ethacrynic acid. However, we have found no evidence in mice to support this view since the oral $LD_{50}$ in Tuck/TO female mice was similar to that in Tuck/TO male mice. It is possible that ethacrynic acid is metabolized to some other more active derivative which is not less toxic in this species.

The nature of the renal receptor for ethacrynic acid is unknown. Various modes of action have been suggested including inhibition of $Na^+K^+$ adenosine triphosphatase, adenyl cyclase or glycolysis and displacement of cyclic adenosine 3′,5′-monophosphate from binding sites (Jacobson & Kokko, 1976). The sex difference in response might, therefore, be due to an effect of sex hormone balance on receptor sensitivity but there is as yet no evidence to substantiate this view.

We wish to thank the various Pharmaceutical Companies for gifts of standard diuretic agents and Dr R. Gaunt, Ciba-Geigy Ltd for a supply of Su 15049A. The technical assistance of G.A. Foster and Mrs K.J. Foster is gratefully acknowledged.

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# THE EFFECT OF ANGIOTENSIN I CONVERTING ENZYME INHIBITOR (SQ 20881) ON THE RELEASE OF PROSTAGLANDINS BY RABBIT KIDNEY, *in vivo*

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1 Prostaglandin E- and F-like material has been estimated in renal venous blood of the left kidney of anaesthetized rabbits following renal nerve section. Prostaglandins were estimated by bioassay following solvent extraction and column chromatography.

2 Electrical stimulation of the renal nerves of the left kidney to reduce renal blood flow by approximately 15% for 15 min resulted in a significant increase in the concentration of prostaglandin E-like material in the renal venous blood. The peak values were normally seen either in the last 5 min of the stimulation period or in the first 5 min after the end of the stimulation period. The concentration of prostaglandin F-like material was not significantly altered.

3 Similar reduction of renal blood flow of the left kidney by renal artery constriction also resulted in a significant increase in the concentration of prostaglandin E- but not F-like material in renal venous blood. The timing and magnitude of the response was comparable with that observed with renal nerve stimulation.

4 The effect of an angiotensin I converting enzyme inhibitor, SQ 20881, on the response to both renal nerve stimulation and renal artery constriction has been studied. The administration of the drug did not significantly reduce the release of prostaglandins from the denervated kidneys, however, the increase in prostaglandin E-like material, in response to both stimuli, was abolished.

5 The results suggest that the increase in prostaglandin E-like material released from the kidney in response to low frequency stimulation or to modest reductions in renal blood flow is dependent on the release of renin and that the effect is mediated by the formation of angiotensin II and not angiotensin I.

## Introduction

The renin-angiotensin, prostaglandin and kallikrein systems of the kidney appear to be closely interrelated; however, a clear picture of these relationships has not yet emerged. Infusion of angiotensin II into the renal artery leads to an increase in the release of prostaglandin-like material from the kidney in the dog (McGiff, Crowshaw, Terragno & Lonigro, 1970; Aiken & Vane, 1973) and from the isolated Krebs-perfused kidney of the rabbit (Needleman, Kauffman, Douglas, Johnson & Marshall, 1973). Stimulation of the renal nerves, which leads to the intrarenal generation of angiotensin II and increase in renin release from the kidney (Vander, 1965; Coote, Johns, Macleod & Singer, 1972; Loeffler, Stockigt & Ganong, 1972) has also been associated with an increase in prostaglandin release in the dog (Dunham & Zimmerman, 1970; Mancia, Romero & Strong, 1974) and the rabbit (Davis & Horton, 1972). Another stimulus which leads to an increase in renin release from the kidney is renal artery constriction (Johns & Singer, 1974). This stimulus has also been reported to induce an increase in the release of prostaglandin from the kidney in the dog (McGiff, Crowshaw, Terragno, Lonigro, Strand, Williamson, Lee & Ng, 1970; Gross, Mujovic, Jubiz & Fisher, 1976). Although these results suggest that the increased formation of angiotensin II within the kidney leads to the local formation of prostaglandin, other evidence indicates that the relationship between these substances is very complex, and that the kallikrein-kinin system may also be involved. Larsson, Weber & Änggård (1974) have shown that administration of arachidonic acid in rabbits led to an increase in plasma renin activity (PRA), while indomethacin, which inhibits prostaglandin synthesis, led to a significant decrease in PRA. The dog also responds to infusion of arachidonic acid with an increase in renin secretion which has been attributed to a direct action of prostaglandin on the juxtaglomerular cell (Bolger, Eisner, Ramwell & Slotkoff, 1976). When indomethacin or meclofenamate were used to block the synthesis of prostaglandin, the rise in

PRA in the rabbit, in response to haemorrhage or furosemide administration, was diminished (Romero, Dunlop & Strong, 1976). In man, PRA was reduced after oral administration of indomethacin (Donker, Arisz, Brentjens, van der Hem & Hollemans, 1976).

McGiff, Itskovitz, Terragno & Wong (1976), in studies based on the administration of kinins, have suggested that kinins increase prostaglandin synthesis by making more substrate available to the prostaglandin synthetase complex. In a study of the isolated Krebs-perfused rabbit kidney, bradykinin added to the perfusion fluid augmented the release of a prostaglandin E-like substance, while aprotonin, a kallikrein inhibitor, reduced it. Addition of a kininogen had the same effect as bradykinin (Nasjletti & Colina-Chourio, 1976). On the other hand Obika & Mills (1976) found that arterial infusion of prostaglandin E in dogs leads to an increase in urinary excretion of kallikrein, and they suggest that the diuresis and natriuresis associated with this treatment may be secondary to the release of kallikrein.

The present study is an attempt to examine the relationship between the release of renin and the release of prostaglandins from the kidney, *in vivo*, in response to moderate stimuli, i.e. stimulation of the renal nerves and renal artery constriction, to reduce renal blood flow by about 15%. These experiments were performed on denervated kidneys of anaesthetized rabbits, and the prostaglandin content of renal venous blood was examined before, during and after the stimulus was applied. The experiments were also performed after the administration of the angiotensin I converting enzyme inhibitor (CEI), SQ 20881, which blocks the conversion of angiotensin I to angiotensin II and which inhibits kininase, thus potentiating the effect of locally generated kinins (Engel, Schaeffer, Gold & Rubin, 1972; Erdös, 1975).

It was hoped that this approach might avoid some of the problems associated with isolated organs, especially when perfused with synthetic media, or with the administration of substances not normally reaching the kidney via the renal artery. As SQ 20881 blocked the formation of angiotensin II while preventing the breakdown of kinins the experiments were expected to give some information about the relative importance of these substances in mediating the release of prostaglandins in response to the stimuli under investigation.

## Methods

### *Preparation of animals*

New Zealand Red rabbits of either sex (2.1–3.2 kg) were anaesthetized with intravenous sodium pentobarbitone (marginal ear vein), and the trachea was cannulated as well as a jugular vein and a carotid artery. The left kidney was exposed via a retroperitoneal incision. The renal nerves were sectioned and a flow probe placed on the renal artery (Biotronix, 1.25 mm) and connected to a flow meter (S.E. Laboratories M275). Renal blood flow was recorded on a Grass Polygraph. The lumboadrenal vein was cannulated and the tip of the cannula was manoeuvered into the renal vein. After surgery was completed heparin (1000 u/kg) was administered.

### *Collection of renal venous blood samples*

At least 1 h was allowed to elapse following renal denervation before the first renal venous blood sample was collected. Each renal venous blood sample (3–5 ml) was collected into a cooled 5 ml plastic syringe over a 5 min period. During this period about one half to three quarters of the blood volume removed was replaced with dextran (Lomodex 70, Dextran, Fisons Ltd.) intravenously. The blood was placed in a graduated test tube surrounded by ice and extracted immediately.

### *Extraction of blood*

Immediately after collection, 0.1 ml of ethanol containing approximately 0.01 µCi [n-5, 6, 8, 11, 12, 14, 15-$^3$H]-prostaglandin $E_2$ (specific activity 120–170 Ci/mmol, Radiochemical Centre, Amersham), was added to the blood and mixed. The blood was lysed with an equal volume of ice-cold deionized water. One half of the diluted blood volume of 0.5 molar sodium phosphate buffer at pH 5.0 containing 10 mg/ml L-ascorbic acid was added. The ascorbic acid acted as an anti-oxidant (Edwards, Strong & Hunt, 1969) and significantly improved extraction recoveries. The lysed blood in phosphate buffer was extracted with 40 ml of 5% methanol in dichloromethane. Following centrifugation at 0°C the organic solvent phase was decanted off and dried down under vacuum on a rotary evaporator (40–45°) and stored under nitrogen. This extraction procedure is a modification of the method of Golub, Zia & Horton (1974).

### *Column chromatography*

The method used is a modification of the method of Caldwell, Burstein, Brock & Speroff (1971). Prostaglandin E- and F-like substances were separated using silicic acid plus celite microcolumns loaded with methanol. The methanol was removed with benzene:ethyl acetate (60:40) and the extracts (or standards) were applied to the column in benzene:ethyl acetate:methanol (60:40:10).

Prostaglandins E and F were eluted from the column by increasing the proportion of methanol in the eluting solvent. Sixteen column fractions were collected, each of 6 ml, the solvent being forced through the column under pressure to produce a flow rate of 1 ml/minute. Column fractions 1–3 were eluted with benzene:ethyl acetate (60:40). These column fractions were discarded since they contained prostaglandins A and B and other unwanted components. Column fractions 4–12 were eluted with benzene:ethyl acetate:methanol (60:40:5). Prostaglandin E characteristically appeared over a well-defined peak, occurring in column fractions 4–7. For routine experiments these were the only column fractions kept, the rest were discarded. Column fractions 12–16 were eluted with benzene:ethyl acetate:methanol (60:40:20). This was the prostaglandin F zone.

Corrections for losses were made by counting 1 ml aliquots of column fractions 4–7 (prostaglandin E) and 12–16 (prostaglandin F) for each sample. The pooled total counts found in the four prostaglandin E fractions (about 30%) were used to determine the losses which occurred during the extractions and purification procedures. The estimate of loss found in the prostaglandin E zone was also applied to the prostaglandin F values as it had been confirmed by bioassay that the losses of both prostaglandins in any one extract were usually similar. The prostaglandin F values were also corrected for a small contamination with prostaglandin E (assessed by radioactivity and usually less than 5%), taking into account the relative potencies of prostaglandins E and F in the bioassay.

### *Bioassay*

For bioassay the column fractions for each prostaglandin (5 ml of each) were pooled and the solvent evaporated with a jet of nitrogen and the fractions stored in the deep freeze. The extract was taken up in 1 ml Tyrode solution and assayed against either prostaglandin $E_2$ or $F_{2\alpha}$ on the rat isolated stomach strip (Vane, 1957). Indomethacin, 1–10 μg/ml, was added to the Tyrode solution to inhibit spontaneous activity and increase the sensitivity of the tissue. To eliminate effects due to other substances such as acetylcholine, histamine, 5-hydroxytryptamine and the catecholamines, the following blocking agents were routinely added to give final bath concentrations/ml as follows: atropine 0.1 μg; diphenydramine 0.1 μg; bromo-lysergic acid diethylamide 0.2 μg; phenoxybenzamine 1.0 μg (Sirois & Gagnon, 1974).

### *Renal nerve stimulation*

The distal cut ends of the renal nerves were placed over silver wire stimulating electrodes for direct electrical stimulation. A 15-min period of renal nerve stimulation was employed, with the intensity adjusted to maintain a 15% reduction in renal blood flow. Square wave stimuli were delivered from a Grass S8 stimulator. Stimulation parameters were 15 V, 0.2 ms duration and, usually, 1 to 1.5 hertz.

### *Renal artery constriction*

A cotton thread was passed around the renal artery and through a length of polythene tubing attached to a screw device which could be raised or lowered to adjust the degree of constriction applied. Blood flow was reduced by 15% for a period of 15 minutes.

### *SQ 20881 (Pyr – Trp – Pro – Arg – Pro – Glu – Ile – Pro – Pro)*

This was made up as required as a 3 mg/ml solution in 0.9% w/v NaCl solution (saline). Doses of 1.5 mg were administered every 15 min in the first few experiments, and every 10 min in the rest in an attempt to maintain a reasonable degree of converting enzyme inhibition. As data were not available on the rabbit the dose level and timing were chosen on the basis of published data for the rat (Engel *et al.*, 1972).

### *Experimental design*

The maximum number of renal vein blood samples removed from any one animal was six. Two basic experimental protocols were used in the experiments. (1) Six samples were taken during a single experiment, 2 before stimulation, 2 during the last 10 min of a 15 min period of stimulation (either renal nerve stimulation or renal artery constriction), and 2 during consecutive 5-min periods immediately after the end of stimulation. It was found that the prostaglandin content of 6 renal venous blood samples, taken in this manner, but without the stimulus, were almost identical. (2) Six samples were taken during 2 experiments; one experiment performed before and one during the administration of SQ 20881. Three samples were removed during each experiment; one before stimulation, one during the last 5 min of a 15-min period of stimulation and one during the first 5 min immediately after the end of the stimulation. It was found that, in the absence of the SQ 20881, the response in the second experiment was similar to the first, with both stimuli.

Statistical analysis was performed using Student's *t* test.

## Results

### *Renal nerve stimulation*

Five experiments were performed in which 6 renal venous blood samples were taken before, during and after a single stimulation period of 15 min (Table 1). The mean prostaglandin E-like material rose from 1.94 ± 0.43 (s.e. mean) ng/ml and 0.93 ± 0.39 during the control periods to 5.6 ± 1.6 ($P < 0.025$) in the immediate post-stimulation period. In the fifth to tenth minute of post-stimulation period the value had returned to 0.98 ± 0.41 ng/ml. In the individual experiments, the peak values occurred in the immediate post-stimulation period in 4 experiments and the 5th–10th min of stimulation period in one experiment. Collections were not made in the first 5 min of the stimulation period as preliminary studies showed that there was no change in prostaglandin levels in this period. Prostaglandin F was not determined in the group of animals.

### *During the administration of SQ 20881*

Five experiments were performed, as above, except that the converting enzyme inhibitor was administered every 15 min (3 experiments) and later every 10 min (2 experiments) until the last sample was removed. The first blood collection period was started immediately after the first injection of the drug. The concentrations of prostagland E- and F-like substances were estimated (Table 1). Prostaglandin E values in the control period were 1.73 ± 0.45 and 1.08 ± 0.18 ng/ml. All of the values after this were within this range, or lower. The prostaglandin E concentration during the last 5 min of stimulation was actually significantly lower than the first control sample, 0.64 ± 0.10 ($P<0.025$). It was not, however, significantly lower than the second control sample ($P>0.05$). The concentrations of prostaglandin F-like material were 1.10 ± 0.49 and 0.66 ± 0.40 ng/ml in the 2 control samples, and the subsequent four values fell within this range.

*Experiments performed before and after the administration of SQ 20881.* Four experiments were performed in which the nerve was stimulated before and after the administration of the drug. In a fifth animal two experiments were performed, but the drug was not administered during the second experiment. These experiments are included in the group which did not receive SQ 20881 (Table 3). The timing of the blood samples was based on the fact that most of the peaks occurred in the immediate post-stimulation blood samples in the previous experiments (first paragraph of Results section). In the experiment performed before the administration of the drug the mean prostaglandin E concentration in the control blood was 2.27 ± 0.70 ng/ml. The mean peak value, found either at the end of the stimulation period, or immediately after this, was 7.32 ± 1.6 ng/ml ($P<0.01$, $n=6$). The peak values were found in the second blood sample in 3 experiments and the third blood sample in 3 experiments. The mean prostaglandin F value in the control blood was 1.00 ± 0.47 ng/ml. The peak value associated with the renal nerve stimulation was 1.53 ± 0.91 ($P>0.15$, $n=4$).

After the administration of SQ 20881, the prostaglandin E concentration was 1.03 ± 0.31 ng/ml before stimulation and was not significantly different in the two subsequent blood samples. The prostaglandin F concentration was 0.77 ± 0.30 before stimulation and was not significantly different in the next two samples.

*Pooled data.* (Figure 1) In the 11 experiments in which the renal nerve was stimulated in the absence of the blocker, the mean prostaglandin E concentration in the renal venous blood before stimulation was 2.12 ± 0.41 ng/ml. The mean peak value was 6.62 ± 1.12 ($P<0.025$).

After the administration of SQ 20881 the prostaglandin E concentration before stimulation was 1.42 ± 0.30 ng/ml and the peak value was 1.51 ± 0.37 ($n=9$, $P>0.4$). The values for prostaglandin F were 0.96 ± 0.29 before stimulation and the peak value was 1.48 ± 0.42 ($P>0.1$, $n=9$).

**Table 1** Effect of renal nerve stimulation on prostaglandin-like material in renal venous blood, in the absence and presence of angiotensin I converting enzyme inhibitor (SQ 20881)

| *Time (min)* | *Control*<br>0–5 | *Control*<br>10–15 | *Stim*†<br>25–30 | *Stim*††<br>30–35 | *Post*<br>35–40 | *Post*<br>40–45 |
|---|---|---|---|---|---|---|
| PGE conc. ng/ml ± s.e.mean $n=5$ | 1.94 ± 0.43 | 0.93 ± 0.39 | 1.80 ± 0.91 | 2.19 ± 0.80 | 5.60 ± 1.60* | 0.98 ± 0.41 |
| Admin. of SQ 20881. PGE conc. ng/ml ± s.e. mean $n=5$ | 1.73 ± 0.45 | 1.08 ± 0.18 | 0.90 ± 0.39 | 0.64 ± 0.10* | 1.38 ± 0.59 | 0.88 ± 0.44 |
| Admin. of SQ 20881. PGF conc. ng/ml ± s.e. mean $n=5$ | 1.10 ± 0.49 | 0.66 ± 0.40 | 0.92 ± 0.48 | 0.92 ± 0.60 | 0.66 ± 0.50 | 0.72 ± 0.72 |

† 5th to 10th min of stimulation; †† 10th to 15th min of stimulation.
* $P<0.025$ when compared with first control value.

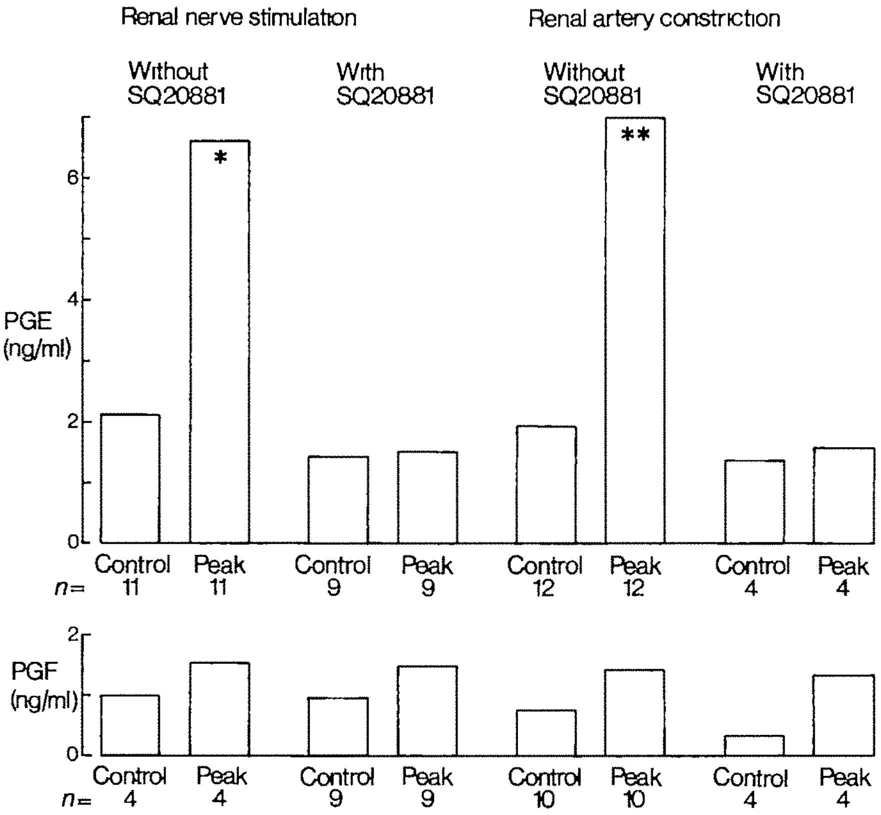

**Figure 1** Summary of data from Tables 1 to 3. When two control values were available the first value was used. The peak value refers to the highest value obtained during or immediately after the application of the stimulus.
$^*P<0.025$, $^{**}P<0.0025$.

*Renal artery constriction*

Six experiments were performed in which 6 renal venous blood samples were taken before, during and after a single 15-min period of renal artery constriction which lowered renal blood flow by 15% (Table 2). The mean prostaglandin E-like material was 1.60±0.85 and 1.60±0.50 ng/ml in the control periods, and it rose to a peak of 7.7±2.65 ($P<0.025$) during the last 5 min of the constriction period. It was still significantly increased in the first post-constriction period (3.90±0.87, $P<0.025$) but fell to slightly under the control values in the second post-constriction period, 1.09±0.39. In the individual

**Table 2** Effect of renal artery constriction on the concentration of prostaglandin-like material in rabbit renal venous blood

| *Time (min)* | *Control* 0–5 | *Control* 10–15 | *Constr†* 25–30 | *Constr††* 30–35 | *Post* 35–40 | *Post* 40–45 |
|---|---|---|---|---|---|---|
| PGE conc. ng/ml ±s.e. mean $n=6$ | 1.60±0.85 | 1.60±0.50 | 3.10±1.27 | 7.70±2.65* | 3.90±0.87* | 1.09±0.39 |
| PGF conc. ng/ml ±s.e.mean $n=6$ | 1.12±0.31 | 0.55±0.24 | 1.24±0.54 | 0.80±0.22 | 0.88±0.46 | 0.50±0.32 |

†5th to 10th min of renal artery constriction; ††10th to 15th min of renal artery constriction.
* $P<0.025$ when compared with first control value.

experiments, the peak occurred in the last 5 min of the artery constriction in 4 experiments, and in the immediate post-stimulation period in two. The mean prostaglandin F-like material in the blood taken during the control periods was $1.12 \pm 0.31$ and $0.55 \pm 0.24$ ng/ml and it was not significantly different in the subsequent 4 blood samples.

*Experiments performed before and after the administration of SQ 20881.* Four experiments were performed in which the renal artery was constricted both before and after the administration of SQ 20881. The drug was administered every 10 minutes. In a fifth animal 2 experiments were performed in which the drug was not administered during the second experiment. These experiments are included in the group which did not receive the SQ 20881 (Table 3).

In the experiments performed without inhibitor, the concentration of prostaglandin E-like material was $2.27 \pm 0.79$ ng/ml in the control period. The mean peak value obtained during or immediately after the constriction was $5.5 \pm 1.34$ ($P<0.05$). Before the administration of SQ 20881, the mean concentration of prostaglandin F-like material was $0.75 \pm 0.48$ ng/ml and it was not significantly altered by the stimulus.

After the administration of the converting enzyme inhibitor, the concentration of prostaglandin E-like material in the renal venous blood was $1.40 \pm 0.56$ ng/ml in the control period. The mean peak value was $1.60 \pm 1.01$ ($P>0.4$). The concentration of prostaglandin F-like material in the equivalent periods was $0.35 \pm 0.22$ in the control period and $1.35 \pm 0.78$ for the peak value ($P>0.1$).

*Pooled data.* (Figure 1) a total of 12 experiments was performed in which the renal artery was constricted in the absence of the blocker. In the control period before constriction the concentration of prostaglandin E-like material in the blood was $1.96 \pm 0.56$ ng/ml. The peak value during or immediately after constriction was $7.02 \pm 1.34$ ($P<0.0025$). The comparable values for prostaglandin F-like material (10 experiments) was $0.76 \pm 0.25$ and the peak value during or immediately after constriction was $1.43 \pm 0.42$ ($P>0.05$).

*Effect of SQ 20881 on basal levels of prostaglandins E and F in renal venous blood*

Using the combined data from all the experiments (the first control value when two were obtained) the mean concentration of prostaglandin E-like material in control blood samples was $2.02 \pm 0.35$ ng/ml ($n=23$). After the administration of the SQ 20881 the mean concentration of prostaglandin E-like material was $1.55 \pm 0.24$ ($n=13$, $P>0.15$). For prostaglandin F, the values were $0.83 \pm 0.21$ ($n=14$) before the administration of the blocker and $0.77 \pm 0.22$ ($n=13$) after the administration of the drug ($P>0.4$).

## Discussion

The present experiments indicate that it is possible to detect small amounts of prostaglandin E- and F-like material in the renal venous blood of denervated kidneys of anaesthetized rabbits. Stimulation of the renal nerves for 15 min, to reduce renal blood flow by about 15%, resulted in a significant increase in the concentration of prostaglandin E-like material in the renal venous blood. We believe this reflects an increase in the intrarenal generation of prostaglandin E-like material. The actual secretion rate would be closely related to these changes in concentration, however, the values would require correction for the small decrease in renal blood flow during the stimulation period and

**Table 3** Effect of renal nerve stimulation or renal artery constriction on prostaglandin release from the kidney before and after the administration of angiotensin I converting enzyme inhibitor in the same animal

| | *Renal nerve stimulation* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Before administration of SQ 20881* | | | | *After administration of SQ 20881* | | | |
| | n | *Control* | *Peak* | *P value* | n | *Control* | *Peak* | *P value* |
| PGE conc. ng/ml ± s.e. mean | 6 | 2.27 ± 0.70 | 7.32 ± 1.60 | < 0.01 | 6 | 1.03 ± 0.31 | 1.55 ± 0.59 | > 0.4 |
| PGF conc. ng/ml ± s.e.mean | 4 | 1.00 ± 0.47 | 1.53 ± 0.91 | > 0.15 | 4 | 0.77 ± 0.30 | 1.63 ± 0.55 | > 0.1 |
| | | | | *Renal artery constriction* | | | | |
| PGE conc. ng/ml ± s.e. mean | 6 | 2.27 ± 0.79 | 5 50 ± 1.34 | < 0.05 | 4 | 1 40 ± 0.56 | 1.60 ± 1.01 | > 0.4 |
| PGF conc. ng/ml ± s.e. mean | 4 | 0.75 ± 0.48 | 0.70 ± 0.42 | > 0.45 | 4 | 0.35 ± 0.22 | 1.35 ± 0.78 | > 0.1 |

the small increase in renal blood flow, above the control level, in the immediate post-stimulation period. As the peak of the response was found at the end of the stimulation period in about half of the experiments, and in the immediate post-stimulation period in the other half, the mean increases in concentration recorded in this study closely approximate changes in secretion rate. The present studies support earlier work on the effect of renal nerve stimulation, under slightly different experimental conditions (Dunham & Zimmerman, 1970; Davis & Horton, 1972; Mancia *et al.*, 1974).

Renal artery constriction, to reduce the renal blood flow to the same extent as with neural stimulation, also resulted in a significant increase in the release of prostaglandin E-like material which was similar in magnitude and in timing to that which occurred with neural stimulation. This is in general agreement with the reports of McGiff *et al.* (1970), who constricted the renal artery to reduce renal blood flow by 50%, and of Gross *et al.* (1976), who reduced renal blood flow by 30%, as compared with 15% in the present study.

We chose both of the stimuli used in the present study because we had previously observed, in the cat, that these manoeuvres induced the release of renin from the kidney. The mechanisms by which these stimuli produce their effect on renin release are different, as it is possible to block the effect of neural stimulation with propranolol, but not the effect of renal artery constriction (Johns & Singer, 1974). The present results with the angiotensin I converting enzyme inhibitor suggest that the generation of angiotensin II is necessary in order to demonstrate the increase in the release of prostaglandin E-like material from the kidney, regardless of the stimulus. They support the results of *in vivo* studies by Satoh & Zimmerman (1975) who reported that the effect of prostaglandin synthetase inhibitors on renal blood flow was greater when renin release was high, e.g. during partial renal artery occlusion, than under basal conditions, and that pretreatment with an angiotensin antagonist (1-sar-8-ala-angiotensin II) led to a marked inhibition of the effect of the prostaglandin synthetase inhibitors. The importance of the renin/angiotensin system in mediating the production of prostaglandin following neural stimulation might be queried because this stimulation leads to the release of noradrenaline, and it has been reported by McGiff, Crowshaw, Terragno, Malik & Lonigro (1972) that intrarenal infusion of noradrenaline leads to the release of prostaglandin from the kidney. However, as the angiotensin I converting enzyme inhibitor blocked the response, it would appear that the noradrenaline released in response to renal nerve stimulation in the present experiments was probably having its effect on prostaglandin through its renin-releasing effect. Thus, not only does blockade of prostaglandin production *in vivo* prevent the release of renin induced by a physiological stimulus such as haemorrhage (Romero *et al.*, 1976) but also the blockade of angiotensin II generation in response to the two physiological stimuli of the present study prevent the increase in prostaglandin release. How this apparent see-saw mechanism works is not clear.

Although there is much evidence that administration of kinins or kinogens leads to increased prostaglandin E release from the kidney, the present results do not support the idea that the generation of prostaglandin in the kidney is dependent on the local generation of kinins. The blocker used in these experiments, SQ 20881, is a more potent blocker of kininase than of angiotensin I converting enzyme (Engel *et al.*, 1972). Thus it is likely that if the stimuli which lead to the release of prostaglandin did so by increasing the formation of kinins which then mediated the prostaglandin response, the levels achieved, upon stimulation should have been increased. As this did not occur, it seems more likely that the increased generation of prostaglandin in response to these two stimuli is not directly dependent on the local concentration of kinins. However, it is possible that kinins may have a permissive effect on the prostaglandin response and that preventing their breakdown does not further affect their influence. The effect of aprotonin, which would block the formation of kinins, might resolve this point.

Several recent studies have indicated that renal artery constriction leads to a fall in urinary kallikrein in the dog (Keiser, Andrews, Guyton, Margolius & Pisano, 1976; Mills, Macfarlane, Ward & Obika, 1976). Keiser *et al.* (1976) report a correlation between urinary kallikrein and renal blood flow while Mills *et al.* (1976) stress the relation between renal artery pressure and urinary kallikrein. They suggest that kallikrein release is dependent on a transmural pressure-sensitive system sited beyond the afferent arteriole. The fall in transmural pressure which occurs during renal artery constriction would then lead to a decrease in the generation of kinins. If this interpretation is correct, kinin production is falling in a situation in which renin and prostaglandin are rising. As renal nerve stimulation should also lead to a fall in transmural pressure beyond the afferent arteriole, a fall in the generation of kinins should also occur under these conditions. Evidence on the effect of renal nerve stimulation on urinary kallikrein excretion is lacking. However, if kinin generation is dependent on a transmural pressure sensitive system as postulated by Mills *et al.* (1976) it is highly unlikely that kinins are involved in mediating the release of prostaglandin E during either renal artery constriction or renal nerve stimulation. The present results, indicating that there is no increase in prostaglandin release in response to the stimuli in the presence of a potent kininase, would support this view. It has recently been reported that plasma bradykinin and plasma renin activity in man are both decreased by saline infusion whereas they are both increased by a postural stimulus, which does not appear to agree with the above suggestion. However, the relation between plasma bradykinin and urinary kallikrein is not clear, and it is possible that the changes in each of these may reflect different phenomena (Wong, Talamo, Williams & Colman, 1975).

The present experiments suggest that the basal level of prostaglandin E-like material released from the denervated kidney is not dependent on the generation of angiotensin II as the concentration was not significantly reduced following the administration of SQ 20881. The present results also indicate that the concentration of prostaglandin F-like substances in renal venous blood are not affected by the generation of angiotensin II either in the basal state or after stimulation of the nerves or after reduction of renal blood flow. Others have found a lack of responsiveness of prostaglandin F in the kidney of the dog (Dunham & Zimmerman, 1970; McGiff *et al.*, 1970). Davis & Horton (1972) did find an increase in prostaglandin F release during renal nerve stimulation in the rabbit, however, they used a much greater intensity of stimulation than was used in the present experiments, i.e. a frequency of 10 Hz as compared with 1 to 1.5 Hz in our studies.

It is apparent that the interrelationship between the renin/angiotensin system and the prostaglandin system is far from clear. In particular, it is not known whether angiotensin influences the synthesis of prostaglandins through its effect on renal haemodynamics or whether it has a direct effect on the prostaglandin producing cells. A recent *in vitro* study, using rat renal papilla, has indicated that angiotensin II will stimulate prostaglandin synthesis by regulating the availability of free precursor (Danon, Chang, Sweetman, Nies & Oates, 1975).

The present results do not, however, confirm several earlier studies in which the isolated Krebs-perfused rabbit kidney was used. In one of these studies, Needleman *et al.* (1973) reported that when angiotensin I was infused into the kidney it caused the release of prostaglandins from the kidney whether it was pretreated with CEI or not. In another study, these workers (Needleman, Douglas, Jakschik, Stoecklein & Johnson, 1974) reported that renal nerve stimulation resulted in prostaglandin release and that this effect was blocked by phenoxybenzamine. Several studies have indicated that renal nerve stimulation of renin release is a $\beta$-adrenoceptor mediated effect and that $\alpha$-adrenoceptor blockade is ineffective (Passo, Assaykeen, Goldfein & Ganong, 1971; Loeffler *et al.*, 1972). This work of Needleman *et al.* (1973; 1974) therefore suggests that renal nerve stimulation on prostaglandin release is not mediated by renin. It also suggests that when renin is involved, the angiotensin I thus formed need not be converted to angiotensin II to be effective. We believe the differences between these results and some of the *in vivo* work is probably related to the use of the unnatural perfusion medium. Such kidneys do not function normally as it has been demonstrated that the isolated Krebs-perfused kidney can neither reabsorb sodium nor concentrate urine (Regoli & Gauthier, 1971). Also, injection of angiotensin I may not produce the same effects as the small quantities of locally generated angiotensin I or II produced in response to a modest stimulus. A repetition of some of the perfusion studies with homologous blood might yield different results.

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# ENZYMATIC HISTAMINE DEGRADATION BY HUMAN SKIN

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1 Degradation of histamine by homogenized human skin *in vitro*, in the presence of the cofactor S-adenosyl methionine, indicates the presence of the histamine metabolizing enzyme histamine-*N*-methyl transferase in human skin. Under the experimental conditions described, no significant histamine degradation by diamine oxidase was observed.

2 The enzyme activity is temperature-sensitive with an optimum at 37°C. The enzyme is stable in intact excised skin at −20°C, but unstable in homogenized skin at this temperature.

3 Little or no enzyme activity is present in mid- or deep dermis, but the distribution of the enzyme between superficial papillary dermis and epidermis is uncertain.

4 The presence of a potent histamine degrading mechanism raises the possibility that histamine-*N*-methyl transferase activity may be an important modulating factor in histamine-mediated skin disorders

## Introduction

The presence of the histamine-degrading enzyme diamine oxidase in guinea-pig skin was recently described (Yamamoto, Francis & Greaves, 1976). We have proposed a role for this enzyme in the regulation of histamine-mediated skin inflammation, since administration of the diamine oxidase inhibitor, aminoguanidine, caused amplification of the passive cutaneous anaphylaxis reaction in this species. Histamine is a mediator of a variety of common inflammatory dermatoses in man. The presence of a histamine catabolizing mechanism in human skin might, therefore, be an important modulating factor in these skin disorders. In this paper we report the presence and preliminary characterization of histamine degrading enzyme activity in human skin.

## Methods

### *Human skin*

Clinically healthy skin removed from the breast, arm, face or abdomen during radical mastectomy or plastic surgery was used. Unless otherwise stated, the skin was stored intact at −20°C, until used.

### *Skin homogenate*

After the subcutaneous fat was removed by surgical scissors, the skin was cut into square pieces, of about 3 mm, with a blade. The pieces were homogenized with 0.01 M phosphate buffered saline (PBS) at pH 7.3, in an ice bath, with a homogenizer (I.L.A. X-1020). The homogenate was filtered through cotton gauze. The filtrate was used as a source of histamine-degrading enzyme, the fibrous residue containing little or no activity.

### *Assay of histamine degrading enzyme activity*

The homogenates obtained from human skin contained endogenous histamine, in concentrations ranging from 600 to 3600 ng/ml. The endogenous histamine in the homogenate was used as a substrate for the study of histamine degrading enzyme activity in skin. Aliquots of the homogenate were incubated in stoppered test tubes. The reaction volume was 0.6–0.7 ml and the incubation time 60 min at 37°C unless otherwise stated. All determinations were carried out in duplicate. After incubation, the reaction was terminated by cooling on ice and the reaction mixture was diluted to 5 ml with ice-cold Tyrode solution and boiled for 10 min, followed by bioassay of the histamine content of the sample. The activity of the histamine degrading enzyme was expressed as the absolute amount of histamine degraded in the reaction mixture after incubation. In each experiment, control samples were kept on ice during the period of incubation and were used to determine the initial histamine content of the homogenate. Histamine degradation at 0°C was negligible (see Results section). In experiments designed to show the presence of enzymatic histamine degradation due to histamine-*N*-methyl transferase, the methyl group donor

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S-adenosyl methionine (S-AM), was included in the incubation mixtures (72 nmol per sample, approximately 0.1 mM) (Brown, Tomchick & Axelrod, 1959).

### *Assay of histamine activity*

Histamine was measured by bioassay, on the guinea-pig isolated ileum preparation in the presence of atropine 1 µM. Each bioassay was carried out in duplicate. In experiments in which aminoguanidine was included in the incubation mixtures, the concentration was equalized in all samples before bioassay, as well as in the histamine standard solutions and Tyrode wash solution.

### *Thin-layer chromatography (t.l.c.)*

Confirmation of the identity of histamine degrading enzyme activity was obtained by thin layer chromatography of the products of [$^{14}$C]-histamine catabolism by skin. A skin homogenate was prepared from 3.25 g of skin in 10.8 ml PBS; 0.5 ml aliquots of the homogenate were added to 6 tubes on ice. To each tube was added 0.19 µCi of [$^{14}$C]-histamine dihydrochloride (equivalent to 84 ng histamine base) and the tubes divided into 3 sets of duplicates. S-AM (72 nmol) was added to one set of duplicates and all the reaction volumes were made up to 0.65 ml with PBS. One set of samples containing S-AM and one set without S-AM were incubated for 60 min at 37°C and the remaining two tubes without S-AM were kept on ice during the incubation, as controls. At the end of the incubation the samples were frozen at −20°C and freeze-dried overnight. To the residues, 300 µl of methanol was added and the tubes thoroughly shaken. Aliquots (150 µl) from each duplicate sample were applied as 3 cm bands on glass t.l.c. plates (200 × 100 × 0.25 mm silica gel G, Anderman). A sample from each duplicate was run on either side of the same plate. Approximately 10 µl aliquots of histamine dihydrochloride, methylhistamine dihydrochloride and imidazole acetic acid hydrochloride solutions (all 2 mg/ml in methanol) were applied at the origin, in the centre of the plate. The plates were then developed to 15 cm in a closed, equilibrated glass tank, using the solvent system chloroform/methanol/ammonia; 70 : 30 : 5 by volume. The plates were then removed, dried and developed a second time in the same solvent system, as this was found to give a better separation of histamine from its metabolites. Dry scrapes (1 cm) of each sample run were washed into scintillation vials, containing 8 ml of scintillant, with 5 ml of methanol and counted on an L.K.B. counter for 300 seconds. The remaining third of each t.l.c. plate was developed in an iodine tank and the $R_F$ values of histamine, methylhistamine and imidazole acetic acid determined.

To compare the degradation of [$^{14}$C]-histamine observed using t.l.c. with that measured by bioassay, a further set of three duplicate samples was set up, using the same homogenate. The samples were prepared and incubated as for the t.l.c. samples, except that 84 ng of histamine base was added instead of [$^{14}$C]-histamine. The histamine content of the samples was then determined by bioassay.

### *Solvents and chemicals*

All solvents were of analytical grade or redistilled before use. The chloroform was redistilled over anhydrous $CaCl_2$ and 1% (by vol.) of absolute ethanol was added to the redistillate to stabilize it. The scintillant for liquid scintillation counting was composed of: toluene/ethoxyethanol, 1500 : 900, v/v; PPO/naphthalene, 10.5 : 112.5, by weight.

[U-$^{14}$C]-histamine dihydrochloride (specific activity 270 mCi/mmol) was obtained from The Radiochemical Centre, Amersham. The following commercial chemicals were also used: histamine dihydrochloride (Koch-Light Laboratories Ltd.), 1-methylhistamine dihydrochloride (Calbiochem Ltd.), imidazole-4-acetic acid hydrochloride, S-adenosyl-L-methionine chloride and aminoguanidine hemisulphate (Sigma London Chemical Company Ltd.), histamine acid phosphate (BDH).

## Results

### *Degradation of histamine by homogenates of human skin*

In 3 experiments samples of separate homogenates were incubated in the absence of S-AM for periods ranging from 15–60 minutes. The results are given in Figure 1. Degradation of histamine in these samples did not exceed 10% of the total, in any experiment. Additional samples of the same homogenates were incubated in the presence of S-AM. Substantial degradation occurred in samples to which S-AM had been added (Figure 1). Prolonging the incubation from 15 min to 60 min caused a progressive increase in the amount of histamine degraded. S-AM was therefore included in the incubation mixture in all subsequent experiments.

### *Aminoguanidine*

In order to exclude the possibility that diamine oxidase contributed to the observed degradation of histamine by the skin homogenate, the experiments were repeated in the presence of the specific inhibitor of diamine oxidase, aminoguanidine, (Blaschko, Friedman, Hawes & Nilsson, 1959) in concentrations ranging from 1 to 100 µM. The incubation period was 60 minutes. Aminoguanidine caused little or no reversal of histamine degradation in the presence of

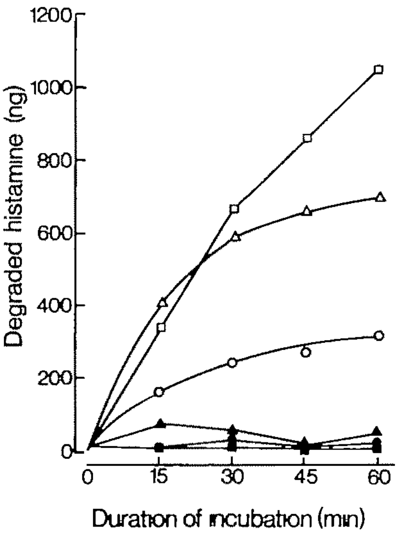

**Figure 1** The effect of S-adenosyl methionine (S-AM) on the time course of the degradation of histamine in samples from 3 separate homogenates of human skin. All homogenates were produced by homogenizing skin in 4 volumes of PBS (w/v). The curves (○), (□) and (△) show the degradation of endogenous histamine in samples, containing 443 ng, 1800 ng and 742 ng of histamine respectively, in the presence of S-AM Curves (●), (■) and (▲) show the corresponding values when samples were incubated without S-AM.

S-AM. There was no significant inhibition by aminoguanidine of the slow degradation of histamine in the absence of S-AM.

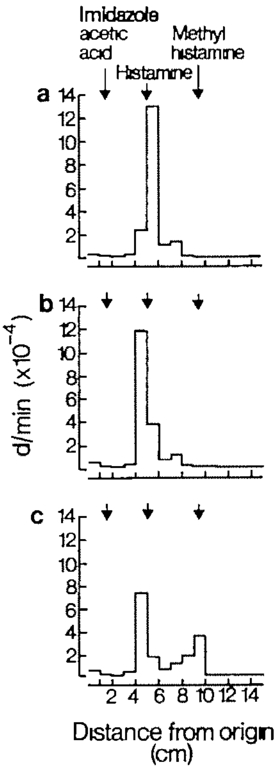

**Figure 2** Thin-layer chromatographic separation of histamine catabolites produced by incubation of [$^{14}$C]-histamine with human skin homogenate All reaction mixtures contained homogenate and [$^{14}$C]-histamine, but only in (c) was S-adenosyl methionine (S-AM) added. The reaction mixtures in (b) and (c) were incubated for 60 min at 37°C, (a) being kept on ice during the incubation to serve as control. Each histogram shows the average profile of duplicate samples run on the same t.l.c. plate. The mean distances run by authentic histamine and its catabolites, methylhistamine and imidazole acetic acid, are indicated by arrows.

### *Thin-layer chromatography*

Figure 2 shows that the two major catabolites of histamine, methylhistamine and imidazole acetic acid can be separated on t.l.c. using the solvent system described in the Methods section. The mean $R_F$ values, for histamine, methylhistamine and imidazole acetic acid were 0.34, 0.63 and 0.11 respectively. The percentage of the total recovered radioactivity which ran with histamine (between 4 and 6 cm) plus methylhistamine (between 8 and 10 cm) on the t.l.c. plates was between 77.8% and 78.8% for the 3 pairs of samples. The corresponding value obtained, where [$^{14}$C]-histamine alone was run on the same system, was 77.1%. In the samples incubated with S-AM, [$^{14}$C]-methylhistamine represented 29.2% and [$^{14}$C]-histamine 48.6% of the total radioactivity recovered. From these figures, 37.5% of the [$^{14}$C]-histamine was converted to [$^{14}$C]-methylhistamine, which compares to a total degradation measured by bioassay of 45%, (representing 139 ng loss of histamine activity). In the absence of S-AM, the bioassay measured a 14.7% loss of histamine activity. On t.l.c., no radioactivity co-chromatographed with imidazole acetic acid or methylhistamine in samples incubated at 37°C without S-AM, giving an almost identical profile to samples kept on ice during the incubation. These results confirm that the observed enzymatic degradation of histamine is attributable to histamine-*N*-methyl transferase.

### *Effect of temperature on histamine*-N-*methyl transferase*

In two experiments, samples of skin homogenates from different donors were incubated for 60 min at

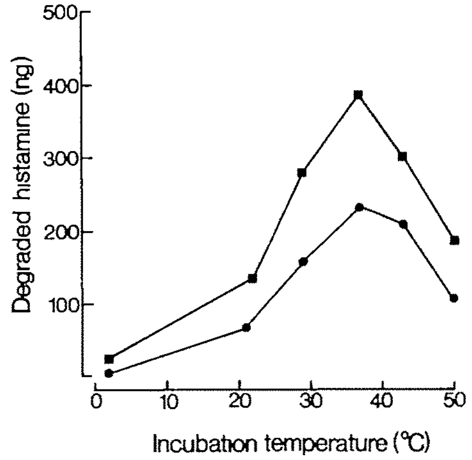

**Figure 3** The effect of temperature on histamine *N*-methyl transferase (HNMT) activity The upper (■) and lower (●) curves show the degradation of histamine in samples, containing 754 ng and 709 ng of endogenous histamine respectively, derived from 2 homogenates of human skin. The skin was homogenized in 3.2 volumes of PBS (w/v) in both cases and all incubations contained S-adenosyl methionine.

temperatures ranging from 2°C to 50°C. The results are shown in Figure 3. Maximum histamine degradation occurred at about 37°C. Increase of the temperature to 43°C caused a drop in activity. Cooling to 29°C caused reduction of histamine degradation and at 2°C degradation was almost completely inhibited.

### *Stability of histamine-*N*-methyl transferase*

There was no detectable loss of enzyme activity in intact skin samples stored at −20°C for up to 27 days. A single sample stored at −20°C for 18 h after homogenization showed a 62.5% decrease in histamine degrading enzyme activity after 60 min incubation with S-AM, with respect to a sample of the same homogenate assayed immediately after homogenization. Therefore, skin was either used fresh or stored intact at −20°C.

### *Localization of histamine-*N*-methyl transferase*

Epidermis was gently scraped from fresh stretched skin, at room temperature, with a blunt scalpel blade. Histological examination of the dermal and putative epidermal fractions revealed that the dermal fraction was devoid of epidermis. The remainder contained the entire epidermis but was also contaminated with elements of the upper papillary dermis. The histamine degrading enzyme activity of these fractions, as assayed in 2 experiments, is given in Table 1. In one experiment there was little or no degradation of histamine by the dermis, although enzyme activity in the other fraction, which contained epidermis as well as elements of upper dermis, was considerable. In the second experiment the results were similar although a small amount of enzyme activity was present in the dermal fraction.

## Discussion

No significant degradation of histamine takes place if fresh homogenized human skin is incubated in the presence of histamine. However, if the methyl donor S-adenosyl methionine (S-AM) is introduced into the reaction mixture, temperature-dependent, progressive histamine degradation occurs, indicating the presence of histamine degrading enzyme activity. Dependence of the reaction on S-AM suggested that the observed degradation was due to histamine-*N*-methyl transferase (Brown *et al.*, 1959). This was confirmed

**Table 1** Values for the degradation of histamine in incubates containing epidermis (plus papillary dermis) and dermis alone, in the presence and absence of S-adenosyl methionine (S-AM).

| | *Experiment 1* | | *Experiment 2* | |
|---|---|---|---|---|
| | *Epidermis* | *Dermis* | *Epidermis* | *Dermis* |
| Total histamine in test samples (ng) | 302 | 219 | 1455* | 868 |
| Histamine degraded in incubate without S-AM | 70 | 19 | 0 | 73 |
| Histamine degraded in incubate containing S-AM | 302 | 2 | 1090 | 160 |

The skin samples in the two experiments shown were from different individuals. Both epidermis and dermis were homogenized in 5 volumes of PBS (w/v). *The total histamine value for samples of epidermis in experiment 2 includes 500 ng of added histamine base. The incubation time for experiment 2 was 90 min and for experiment 1, 60 minutes.

by showing that [$^{14}$C]-methylhistamine was formed from [$^{14}$C]-histamine, when incubated with a human skin homogenate in the presence of S-AM, using thin-layer chromatography.

Two major pathways for histamine catabolism are recognized (Schayer, 1959), catalysed by histamine-*N*-methyl transferase and diamine oxidase respectively. Schayer & Cooper (1956) found that histamine-*N*-methyl transferase was the principal histamine metabolizing pathway in man. We were unable to detect evidence of diamine oxidase activity in human skin, either by t.l.c. or bioassay. T.l.c. of the reaction products of [$^{14}$C]-histamine incubated with a human skin homogenate did not demonstrate the production of any imidazole acetic acid, the final product of the oxidative deamination of histamine. Also, histamine degradation was not inhibited by the specific diamine oxidase inhibitor, aminoguanidine.

Although skin is a major target organ for histamine-mediated allergic reactions, histamine degradation in human skin does not appear to have been studied before. Abdominal skin of the rabbit, mouse and guinea-pig contains significant amounts of histamine-*N*-methyl transferase (Brown *et al.*, 1959) and our own studies (Yamamoto *et al.*, 1976) have shown that guinea-pig skin contains diamine oxidase, as well as histamine-*N*-methyl transferase. It is of interest that no significant metabolism of histamine could be detected in the absence of S-AM. S-AM is a major naturally occurring methyl donor (Baldessarini & Kopin, 1963) formed intra-cellularly from adenosine triphosphate (ATP) and methionine and is used as a methyl group donor by a number of enzymes. The explanation of sub-threshold amounts of this cofactor in the skin homogenates of the present experiments is speculative, but could be explained by failure of *de novo* synthesis after homogenization, together with rapid destruction or use by the several other methylating enzymes which require this cofactor.

The localization of histamine-*N*-methyl transferase in skin requires further study. We were unable to obtain clear separation of epidermis from dermis under conditions that would preserve the activity of the enzyme and at the same time yield sufficient epidermis for study. The method used yielded epidermal samples contaminated with papillary dermis. These samples contained all or almost all enzyme activity. This activity could have derived from epidermis, upper papillary dermis or both. However, there was little or no activity in mid or lower dermis. The closely related enzyme, catechol-*O*-methyl transferase, is present in both epidermis and dermis, although the concentration is higher in epidermis (Bamshad, 1969).

*In vivo* blockade of enzymatic histamine degradation brings about marked enhancement of the passive cutaneous anaphylaxis reaction in the guinea-pig (Yamamoto *et al.*, 1976). Since methylhistamine, the product of the metabolism of histamine by histamine-*N*-methyl transferase, has very little histamine-like biological activity (Lee & Jones, 1949), studies of enzymatic histamine degradation in the skin of patients with urticaria and other histamine-mediated skin disorders, would be of great interest.

We thank Dr C.N. Hensby for his help with thin-layer chromatography. This work was supported by the Medical Research Council. One of us (S.Y.) was in receipt of a research fellowship from the Nuffield Trust, who also provided other financial support.

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# THE EFFECT OF DIURETICS ON THE FAECAL EXCRETION OF WATER AND ELECTROLYTES IN HORSES

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1 The effect on plasma, urinary and faecal electrolytes of frusemide and hydrochlorthiazide was measured in ponies, mean weight 180 kg.

2 The rapid loss in urine of large quantities of sodium had only a small effect on plasma sodium concentration.

3 Faecal sodium excretion was increased substantially after the administration of frusemide.

4 Frusemide increased faecal potassium during the 48 h following administration and faecal water in the 24/48 h period. It also produced a hypopotassaemia.

5 Hydrochlorthiazide increased faecal chloride during the 24 h after administration.

6 Frusemide increased the intestinal transit time of both liquid (polyethylene glycol) and particulate ($Cr_2O_3$) markers.

## Introduction

It was found that the concentration of sodium in the plasma of stabled ponies showed wide fluctuations even in individual animals, and partial sodium depletion did not appear to affect the concentration of this ion in plasma (Alexander, 1974). Usually about three weeks were required to produce sodium depletion by salivary drainage as indicated by the typical change in the sodium/potassium ratio in saliva (Alexander, 1966). Although sodium depletion stimulates aldosterone secretion and thus increases retention of this ion, recent work (Carey, Smith & Ortt, 1976) has shown that, in rabbits, sodium excretion may be controlled through the gastrointestinal tract independent of aldosterone. The purpose of the present experiments was to ascertain whether a more rapid removal of sodium from the pony would change the plasma sodium concentration and, in view of the work of Carey *et al.* (1976) to study the effect on faecal excretion of sodium of the rapid removal of this electrolyte. The rapid excretion of sodium was produced by intravenous injection of frusemide or hydrochlorthiazide.

## Methods

Four male, shetland-type ponies, weight 180 kg ± 8.4 (mean ± s.d.) were used in these experiments. The ponies were trained to stand in metabolism stalls which allowed the collection of urine and faeces (Warwick, 1966). They were fed hay (2.5 kg/day) without concentrates excepting when markers were given. The markers used were chromium oxide (10 g) and polyethylene glycol (200 g) which were thoroughly mixed in about 500 g of bran and oats and fed to the ponies. Frusemide and hydrochlorthiazide were given by injection into the jugular vein. Blood for analysis was taken from the jugular vein into airtight syringes containing either heparin (for blood gas analysis) or ammonium oxalate (for plasma electrolytes). The sample for sodium and potassium determinations was centrifuged and the plasma separated as soon as possible and always within 30 min of bleeding. The sample for pH and blood gas analysis was introduced into a Corning pH/Blood Gas Analyser 165 immediately after withdrawal from the vein. The pH and $P\text{CO}_2$ were determined directly by means of appropriate electrodes, and bicarbonate was calculated automatically from these values. Chloride concentration was measured with an Eel Chloride meter and sodium and potassium by means of a Corning Eel Flame Photometer. Urine was collected over 1 or 2 h periods for the first 6 h and thereafter over 24 h periods for 4 days. Complete faecal collections were made every 24 h for 4 days. The faeces were weighed and thoroughly mixed. A sample of faecal liquor was squeezed from an aliquot for the determination of sodium, potassium and chloride. A

500 g sample of mixed faeces was placed in a vacuum oven and dried to constant weight at below 100°C to ascertain the amount of water excreted in the faeces. The faeces for marker estimations were mixed with twice their weight of water and stirred intermittently for 5 hours. In later experiments mixing time was reduced to 5 min by using a Waring Blender of 5 litre capacity. A sample of the liquor was removed and filtered through No. 42 filter paper. The polyethylene glycol concentration in this filtrate was measured by the method of Hyden (1956) as modified by Smith (1962). Recoveries of polyethylene glycol added to faeces were 96.4 ± 3.2% (mean ± s.e.) for four experiments. The concentration of chromic oxide in dry faeces was determined by the method of Christian & Coup (1954). Recoveries of chromic oxide added to faeces gave values of 101 ± 3.4% (mean ± s.e.) for six experiments. The determinations of sodium and potassium concentration in various fluids were controlled by interposing standard solutions between every three or four samples and the blood gas analyser was standardized before each analysis. The results were analysed using Student's *t* test and the values for plasma sodium concentrations by paired comparisons to obtain additional sensitivity (Sokal & Rohlf, 1973).

The markers were fed together mixed with the bran and oats. Faeces were collected each 24 h period after feeding for three successive days and the amount of each marker excreted per 24 h was determined. Each of the four ponies was fed the markers as a single dose on at least one occasion without and similarly with the administration of frusemide (3.3 mg/kg). The drug was injected as soon as the meal containing the markers had been consumed.

## Results

The effect of frusemide on the concentration of sodium in plasma and the rate of sodium excretion in urine is shown in Figure 1. Although there appears to be a slight fall in the mean plasma value 6 h after

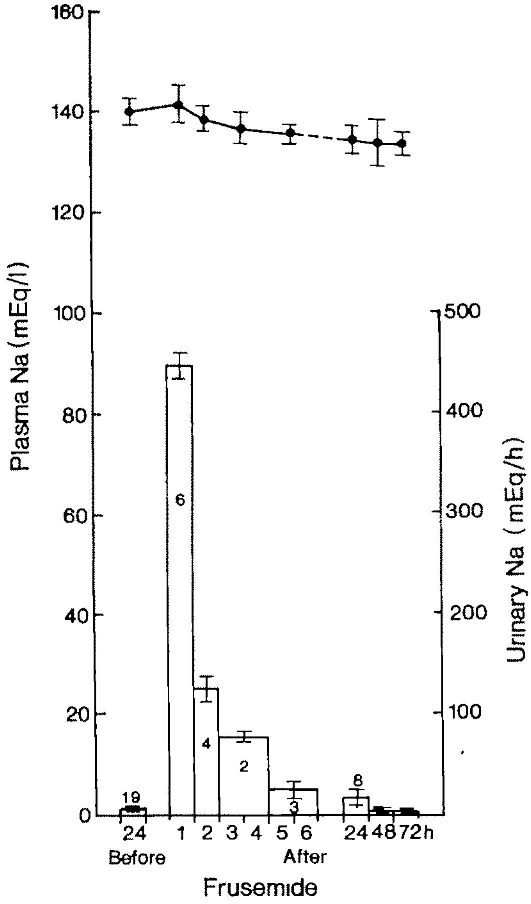

**Figure 1** Effect of frusemide (3.3 mg/kg) on the sodium concentration in plasma and sodium excretion in urine of ponies. The plasma values at 6, 24 and 72 h after treatment differ from the control ($P < 0.1$). Plasma values (●) are shown as mean of eight observations. Vertical lines show s.e. means. Urinary values are shown as mean, number of observations indicated on columns. Vertical lines show s.e. means.

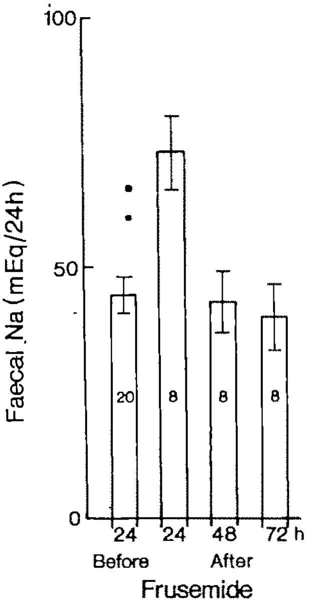

**Figure 2** Faecal sodium excretion per 24 h following frusemide (3 3 mg/kg). Mean values are shown; vertical lines indicate s.e. means Excretion increased during the 24 h following injection ($P < 0.01$). Figures in columns represent number of observations.

giving the drug the mean decrease at the 6th, 24th and 72nd hour was significant only at the $P<0.1$ level. Frusemide treatment (3.3 mg/kg) produced a marked hypopotassaemia ($P<0.02$) within 4–6 h of the drug being injected; the other constituents measured were unaffected, namely chloride, pH, $P\text{CO}_2$ and bicarbonate.

Hydrochlorthiazide (3.3 mg/kg) did not produce a significant change in the plasma sodium, potassium, chloride, bicarbonate, $P\text{CO}_2$ or pH. The diuretic action was less marked than that of frusemide and slower in onset. The sodium excreted in the urine during the 24 h following hydrochlorthiazide was only 1/20th and the urine volume 2/5ths of that produced in the same period by a similar dose of frusemide.

Frusemide produced a marked increase in the faecal excretion of sodium, potassium and water. This is shown in Figures 2, 3 and 4. Although hydrochlorthiazide had a similar effect on faecal sodium when given in the same dose (mg/kg) as frusemide it was significant only at the $P<0.1$ level. However, this dose of hydrochlorthiazide increased the faecal chloride excretion ($P<0.01$) whereas the faecal chloride excretion after a similar dose of frusemide was not significantly changed (Figure 5).

The transit time through the alimentary tract of both a particulate (chromic oxide) and a liquid (polyethylene glycol) marker was increased following the administration of frusemide (Figure 6). Frusemide (3.3 mg/kg) increased faecal weight ($P<0.05$) and water ($P<0.01$) in the second 24 h period after administration whereas hydrochlorthiazide (3.3 mg/kg) increased faecal weight ($P<0.01$) only in the first 24 h following the injection.

## Discussion

Although the rapid removal of a substantial amount of sodium in the urine after frusemide administration appeared to reduce the plasma sodium concentration, the reduction was significant only at the $P<0.1$ level. This was due probably to the wide fluctuations of the

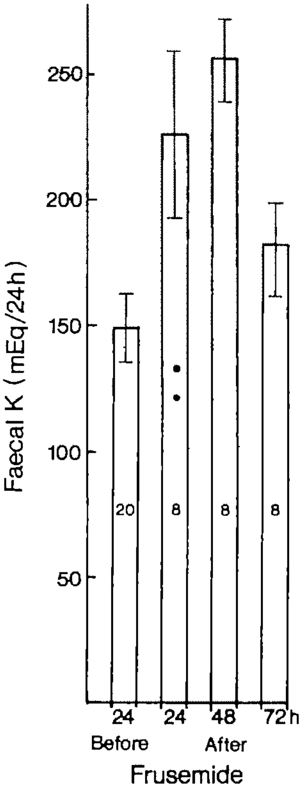

**Figure 3** Faecal potassium excretion increased during the 24 h ($P<0.05$) and, 24/48 h ($P<0.01$) following frusemide (3.3 mg/kg) injections. Mean values are shown; vertical lines indicate s.e. means. Numbers in columns represent number of observations.

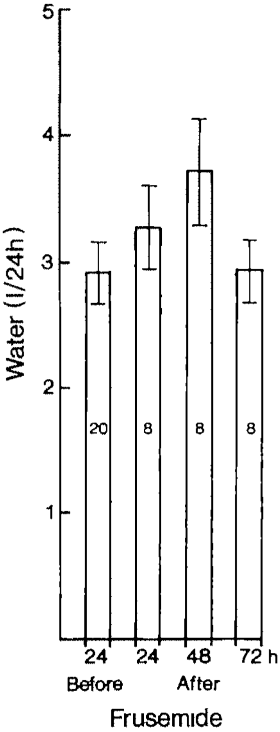

**Figure 4** Increased faecal water excretion ($P<0.01$) produced by frusemide in the 24/48 h period after injection (3.3 mg/kg). Mean values are shown; vertical lines indicate s.e. means. Figures in columns indicate number of observations.

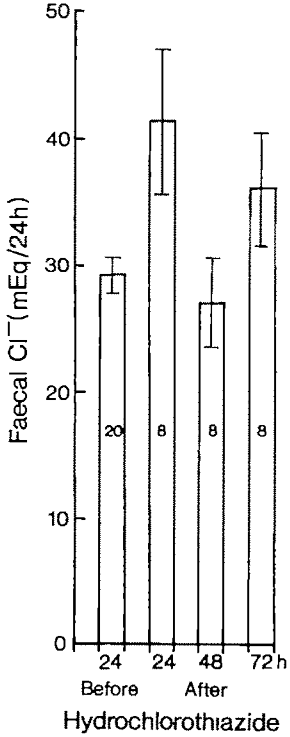

**Figure 5** Increase in faecal chloride excretion ($P<0.01$) in the 24 h following hydrochlorthiazide administration (3.3 mg/kg). Mean values are shown; vertical lines indicate s.e. means. Numbers in columns indicate number of observations

pre-treatment plasma sodium concentration noted earlier (Alexander, 1974) and which has so far defied explanation.

The possibility was considered of the sodium in the liquid contents of the capacious gut providing a reservoir from which the pony could rapidly replenish a deficit in the extracellular sodium. However, the urinary excretion of about 2000 mEq of sodium within 24 h of frusemide injection was accompanied by a significant ($P<0.01$) increase in faecal sodium. Had the sodium in the intestinal liquor been used to compensate for the sodium lost in the urine the faecal sodium would surely have fallen.

Sodium depletion in ponies reduced faecal sodium excretion to about 1/12 of that in replete animals (Alexander, 1966). It was assumed that, as in other species, this increased sodium retention was mediated through the increased secretion of aldosterone. Consequently the urinary sodium loss produced by the diuretics by stimulating aldosterone secretion would, to some extent, have decreased faecal sodium excretion. Despite this the diuretics produced an overall increase in faecal sodium excretion.

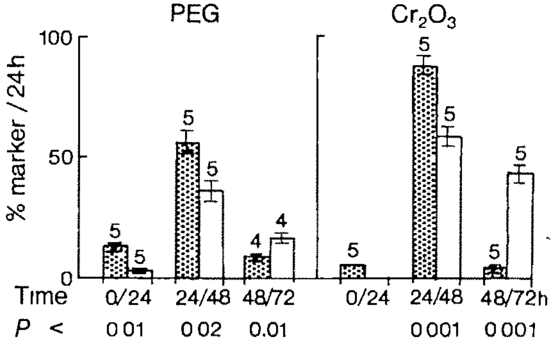

**Figure 6** Excretion of liquid (polyethylene glycol, PEG) and solid ($Cr_2O_3$) markers in faeces. Mean values are shown; vertical lines indicate s.e. means. Numbers on the columns show the number of observations. Stippled columns—no treatment; open columns—frusemide 3.3 mg/kg.

Hydrochlorthiazide differed from frusemide in the greater faecal chloride excretion produced so that when the same dose of each drug was given, on a body weight basis, frusemide did not produce a significant change in faecal chloride whereas hydrochlorthiazide caused an increased ($P<0.01$) faecal excretion of this electrolyte.

In view of the relationship between rate of flow and concentration of salivary sodium (Burgen & Emmelin, 1961) it seemed important to determine whether the increased faecal sodium excretion produced by diuretics reflected a decrease in transit time and a consequent reduction in time for absorption. Since frusemide was the more potent drug, in respect of faecal sodium excretion, studies were limited to this agent.

Argenzio, Lowe, Pickard & Stevens (1974) found differences in transit time through the ponies' gut of various particulate and liquid (polyethylene glycol) non-absorbable markers; therefore in the present experiments transit time was determined using both a liquid (polyethylene glycol) and particulate ($Cr_2O_3$) marker. Frusemide slowed the transit of both markers hence the increased sodium excretion could not be due to the diuretic hastening the transit of intestinal contents through the gut.

It has been shown that the diuretics ethacrynic acid and chlorothiazide inhibited sodium and water transport in isolated sacs of hamster ileum (Binder, Katz, Spencer & Spiro, 1966). More recently Humphreys (1976) found the absorption of sodium chloride from the perfused rat ileum to be inhibited by frusemide. The increased faecal excretion of sodium produced by frusemide and hydrochlorthiazide as described in the present experiments could be due to these drugs impeding the absorption of sodium from the horses' gut. Since the main site of sodium absorption from the gut appears to be the large intestine (Alexander, 1962) this could also be the site of action of the two diuretics. The results of these experiments could be interpreted as supporting evidence of functional similarities between the kidneys and gut which has been accumulating for some time (Binder *et al.*, 1966).

I wish to thank Miss Fiona Anderson for her technical assistance.

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# THE UPTAKE AND OVERFLOW OF RADIOLABELLED β-ADRENOCEPTOR BLOCKING AGENTS BY THE ISOLATED VAS DEFERENS OF THE RAT

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1 A comparison of uptake into and overflow from the isolated vas deferens of the rat has been made between [$^{3}$H]-noradrenaline ([$^{3}$H]-NA), [$^{14}$C]-D-sorbitol and three radio-labelled β-adrenoceptor blocking agents, [$^{14}$C]-practolol, [$^{14}$C]-(±)-propranolol and [$^{3}$H]-penbutolol.

2 The accumulation of [$^{3}$H]-NA after 30 min incubation was reduced by desmethylimipramine (DMI) $1 \times 10^{-8}$M and was also reduced in vasa from rats pretreated with 6-hydroxydopamine (6-OHDA). This was not so with [$^{14}$C]-D-sorbitol.

3 6-OHDA pretreatment of the rats reduced the uptake of [$^{3}$H]-penbutolol after 30 min incubation but not that of [$^{14}$C]-propranolol or [$^{14}$C]-practolol. DMI $1 \times 10^{-8}$M did not alter the tissue uptake of [$^{14}$C]-propranolol, [$^{14}$C]-practolol or [$^{3}$H]-penbutolol.

4 Electrical stimulation of vasa preloaded with [$^{3}$H]-NA caused a significantly greater increase in [$^{3}$H]-NA overflow than during the resting, unstimulated periods. No such increase in overflow was observed with [$^{14}$C]-sorbitol or any of the three β-adrenoceptor blocking agents used.

5 The β-adrenoceptor blocking agent penbutolol was shown to possess adrenergic neurone blocking activity in the isolated vas deferens of the rat.

6 It is concluded that any effect that practolol or (±)-propranolol have on noradrenergic neurones is brought about without the need for these drugs to gain access to the interior of the neurone.

## Introduction

The neurotransmitter noradrenaline (NA) is taken up by noradrenergic nerves (Iversen, 1967). Following electrical stimulation, this stored NA is subsequently released into the surrounding extracellular space (Langer, 1970). These facts are also true for the adrenergic neurone blocking drug guanethidine (Chang, Costa & Brodie, 1964; 1965; Boullin, 1966; Boullin, Costa & Brodie, 1966). Furthermore, the uptake of both NA and guanethidine is reduced by desmethylimipramine (DMI) (Brodie, Chang & Costa, 1965; Iversen, 1967).

The adrenergic neurone blocking action of some β-adrenoceptor blocking agents has been described in some detail by Day, Owen & Warren (1968), Barrett & Nunn (1970), Mylecharane & Raper (1970) and Eliash & Weinstock (1971). Dollery, Lewis, Myers & Reid (1974) have suggested that propranolol is taken up into central noradrenergic neurones via the uptake$_1$ process and that there it causes a release of endogenous NA.

In view of these findings and suggestions, it was decided to investigate the possibility that β-adrenoceptor blocking agents may be taken up into peripheral noradrenergic neurones, as is guanethidine, and that electrical stimulation may cause their release. An attempt has been made to compare the uptake and overflow patterns of some β-adrenoceptor blocking agents with those of the neurotransmitter NA and the extracellularly distributed carbohydrate, D-sorbitol. Studies have also been made on the adrenergic neurone blocking effect of penbutolol.

Some of these results have been presented to the British Pharmacological Society (Lewis, 1974a).

## Methods

### *Uptake studies*

Male Wistar rats weighing 100–120 g were killed by cervical dislocation. Both vasa were removed and immersed in cold modified Holman's solution of the following composition (mM): NaCl 120, $NaHCO_3$ 25, $NaH_2PO_4$ 1, KCl 5, $CaCl_2$ 2.5, glucose 11, sucrose 10, ascorbic acid 0.11 and disodium edetate 0.04. Each vas was stripped of mesentery, blotted on filter paper and weighed. Pairs of vasa were incubated for 5, 10, 20 and 30 min in 2.5 ml of Holman's solution at 37°C gassed with 5% $CO_2$ in $O_2$ and containing one of the following: (a) [$^{14}$C]-sorbitol, $1.4 \times 10^{-5}$M in carrier sorbitol to $3.56 \times 10^{-2}$M; (b) [$^{3}$H]-(−)-NA, $1.23 \times 10^{-8}$M in carrier NA to $2.96 \times 10^{-6}$M; (c) [$^{14}$C]-practolol, $3.15 \times 10^{-5}$M; (d) [$^{14}$C]-(±)-propranolol, $1.52 \times 10^{-5}$M; (e) [$^{3}$H]-penbutolol (Hoe 893d), $8.2 \times 10^{-7}$M.

Each of the above incubations was repeated in the presence of DMI, added to give a final concentration of $1 \times 10^{-8}$M in each medium. Finally, the experiment was replicated at the 30 min incubation time for all media using vasa from rats pretreated with 6-hydroxydopamine (6-OHDA) according to the 14-day schedule of Thoenen & Tranzer (1968).

### *Clearance of [$^{14}$C]-sorbitol*

After incubation of some vasa with [$^{14}$C]-sorbitol for 30 min the tissues were washed for 5–30 min in 50 ml gassed, modified Holman's solution at 37°C which was changed every 5 minutes. Eighty-two per cent of [$^{14}$C]-sorbitol taken up by the tissue was cleared by the first two 5 min washes. It was assumed that this represented clearance of the extracellular space; consequently in the uptake studies where [$^{14}$C]-sorbitol was not used, all tissues were given two 5 min washes in non-radioactive Holman's solution to clear the extracellular space. All results are based on the assumption that 18% of the drug remained in the extracellular space.

### *Sampling and counting*

Following incubation, a 100 µl aliquot of the incubation medium was taken and added to 10 ml of scintillation fluid containing Triton X-100. Vasa were blotted individually, dissolved in 1 ml of Protosol solution at 54°C for 18 h and dispersed in 10 ml of scintillation fluid (without Triton X-100). All samples were counted by standard technique in a SL30 Liquid Scintillation Counter. The accumulation of each drug in the tissue was expressed as counts per minute (ct/min) per mg tissue/ct/min per µl medium, and was termed the 'drug space'. Quenching of samples and efficiency of counting did not vary significantly between samples, therefore ct/min were not corrected to disintegrations per minute.

### *Overflow studies*

Fifteen male Wistar rats weighing 250–500 g were killed by cervical dislocation, the vasa deferatia removed, the mesentery stripped and the organs immersed in cold, modified Holman's solution. After 15 min the tissues were blotted, weighed and incubated for 30 min in modified Holman's solution containing one of the radiolabelled drugs as listed under uptake studies, except for [$^{3}$H](−)-NA where a concentration of $6 \times 10^{-6}$M was used.

Six vasa were used for each incubation. Each vas, after incubation was mounted between two longitudinal platinum wire electrodes of 2 cm length in a 5 ml organ bath and allowed to equilibrate at a tension of 0.3 g for 30 min after [$^{3}$H]NA and for 10 min after the other drugs. After equilibration, the bath fluid was changed and 5 min later 0.5 ml transferred to a scintillation vial, the bath fluid changed again and field stimulation applied 10 s later, for 10 s, using pulses of 1 ms duration, supramaximal voltage, 25 Hz, delivered from an SRI-6053 square wave electronic stimulator. Contractions were recorded with a force displacement gauge coupled to a Devices pen recorder. After 5 min a 0.5 ml sample of the bathing fluid was collected as before. The 5 min periods containing 10 s stimulation were repeated four times.

To each 0.5 ml sample were added 10 ml of Triton scintillant and the sample counted using standard technique. The results were expressed as counts per minute/mg tissue (ct $min^{-1}$ $mg^{-1}$ tissue).

### *Electron microscopy and fluorescence histochemistry*

Morphological evidence for the effectiveness of 6-OHDA-induced sympathectomy in rat vasa has been evaluated by use of electron microscopic and fluorescence histochemical techniques.

Eight male Wistar rats weighing 100–120 g were used for this part of the investigation. Four were treated with 6-OHDA using the 14 day schedule of Thoenen & Tranzer (1968) and four were injected with distilled water to act as controls. At the end of 14 days all animals were killed by cervical dislocation and exsanguination. Both vasa from each rat were prepared for electron microscopic and fluorescence histochemical examination. Standard procedure was used for the preparation of tissues for electron microscopic studies. Glutaraldehyde (5%) and osmium tetroxide (1%) were used as fixatives and after dehydration in ethanol, tissues were impregnated and finally embedded in araldite before sectioning on an LKB ultramicrotome. Sections were contrasted with lead citrate and viewed using a Phillips 300 electron microscope.

Tissue for histofluorescence examination were subjected to the formaldehyde condensation procedure for demonstrating adrenergic nerves by the method of Spriggs, Lever, Rees & Graham (1966). The mounted specimens were viewed under a Zeiss Universal fluorescence microscope.

### *The adrenergic neurone blocking effect of penbutolol*

Vasa from male Wistar rats weighing 250–300 g were set up for transmural stimulation in the manner described for the overflow studies but in a 50 ml bath.

Isotonic contractions were recorded with a lever system coupled to a 'Follograph' (EOS Industrial Electronics Ltd., see Tonks & Williams, 1972). Electrical stimulation of the intramural sympathetic nerve endings was with pulses of 1 ms duration, supramaximal voltage and at frequencies varying from 5 to 20 Hz for periods of 10 s and delivered from an SRI-6053 square wave electronic stimulator.

Responses to NA ($2.96 \times 10^{-5}$M) and to transmural stimulation of various frequencies were recorded in untreated vasa and also 30 min after the addition of several concentrations of penbutolol to the bath.

*Chemicals and drugs*

Scintillation fluid was made up of scintillation grade 2,5-diphenyloxazole 5 g, 4-di(2-(5-phenyloxazolyl) benzene 0.5 g and toluene 1 litre; Triton X-100, 500 ml was also added for aqueous samples. Protosol was obtained from the New England Nuclear Chemical Co. The following drugs were used: desmethylimipramine (Geigy Pharmaceuticals); [$^3$H]-(−)-NA (Radiochemicals, Amersham) specific activity 4.8 mCi/mg for uptake studies and 16.5 mCi/mg for overflow studies; [$^3$H]-penbutolol (Hoechst Pharmaceuticals) specific activity 295.5 µCi/mg; [$^{14}$C]-practolol (ICI Ltd.) specific activity 12.2 µCi/mg; [$^{14}$C]-(±)-propranolol (ICI Ltd.) specific activity 14.66 µCi/mg; [$^{14}$C]-D-sorbitol (Radio chemicals, Amersham) specific activity 9.3 mCi/mg.

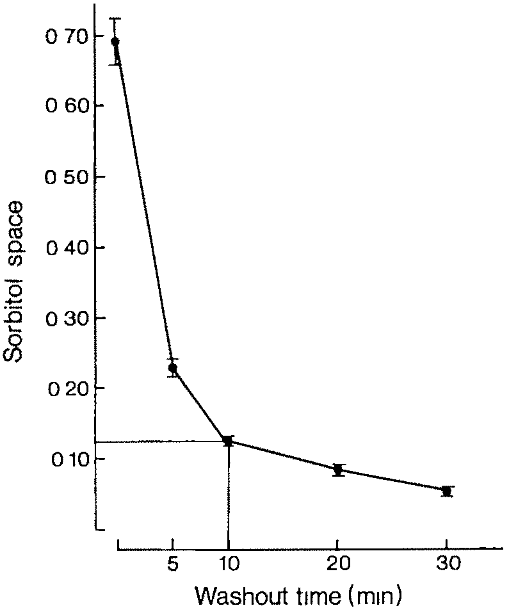

**Figure 1** [$^{14}$C]-sorbitol clearance time from isolated vas deferens of rat. Horizontal axis: Washout times (min); vertical axis: Sorbital space (µl/mg tissue). The graph shows clearance of [$^{14}$C]-sorbital from the isolated rat vas deferens following an initial incubation of 30 min with ]$^{14}$C]-sorbitol; 82% clearance had occurred after two 5 min washout periods.

## Results

*Counting efficiencies*

*The efficiency of counting of* $^{14}$C in Triton scintillant was 92.97±0.61% ($n$=18) and in Protosol scintillant 91.05±0.46% ($n$=18). The efficiencies for tritium were 39.33±0.5% ($n$=18) in Protosol (means±s.e. means).

*Uptake studies*

*Uptake of [$^{14}$C]-sorbitol.* Uptake of [$^{14}$C]sorbitol increased with time, saturation occurring between 20 and 30 min (Table 1). Neither the presence of DMI, $1 \times 10^{-8}$M, nor pretreatment of rats with 6-OHDA affected the uptake pattern. Two 5 min wash periods cleared 82% of the sorbitol from the tissue. (Figure 1).

*Uptake of [$^3$H]-noradrenaline.* The uptake curve for [$^3$H]-NA by the isolated rat vas is shown in Figure 2. There was a rapid accumulation in the initial 5 min followed by a slower phase and saturation had not occurred at 30 minutes. DMI, $1 \times 10^{-8}$M significantly reduced the uptake of [$^3$H]-NA at 30 min (Student's $t$ test, $P<0.02$) but was not effective at 5, 10 or 20 minutes. Pretreatment of rats with 6-OHDA also reduced the uptake of [$^3$H]-NA at 30 min ($P<0.005$) the only time of incubation tested.

*Uptake of [$^{14}$C]-practolol.* The accumulation of [$^{14}$C]-practolol by the isolated vas deferens is shown in Table 1. There was a continual increase in the accumulation of [$^{14}$C]-practolol by the rat vasa at successive incubation times. The amount of [$^{14}$C]-practolol bound by the tissue was less than that of NA at each incubation. The presence of DMI in a concentration of $1 \times 10^{-8}$M did not alter significantly the accumulation curve. Pretreatment of rats with 6-OHDA did not alter significantly the accumulation of [$^{14}$C]-practolol at 30 minutes.

*Uptake of [$^{14}$C]-(±)-propranolol.* The accumulation pattern of [$^{14}$C]-(±)-propranolol is shown in Table 1. There was no evidence of saturation even at the 30 min incubation time. The amount bound by the tissue was greater than that of NA at each incubation time. DMI, $1 \times 10^{-8}$M did not alter the accumulation curve. 6-OHDA pretreatment of the rats did not alter significantly the uptake of [$^{14}$C]-(±)-propranolol at 30 minutes.

*Uptake of [$^3$H]-penbutolol.* Table 1 shows the accumulation of [$^3$H]-penbutolol by the rat isolated vas

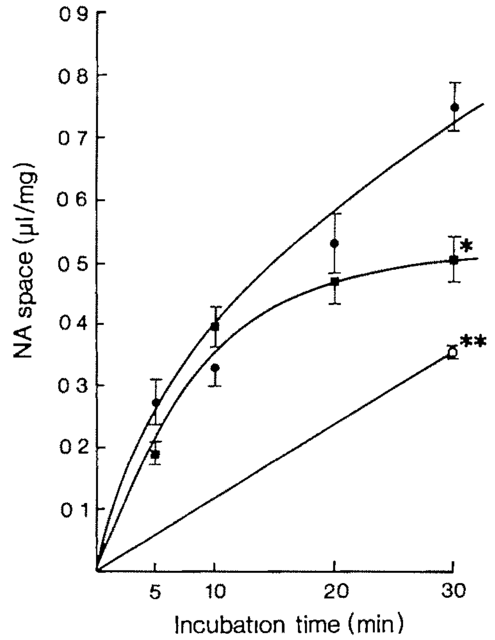

**Figure 2** Uptake of [$^3$H]-(−)-noradrenaline by isolated vas deferens of the rat Horizontal axis: Incubation time (min); vertical axis: noradrenaline (NA) space (µl/mg tissue). (●) Control uptake in untreated rat vasa; (○) uptake in vasa of rats pretreated with 6-hydroxydopamine; (■) uptake of NA in presence of desmethylimipramine 1 × $10^{-8}$ M. Each point is mean of results from a minimum of 4 vasa vertical lines show s.e. mean *$P<0.02$; **$P<0.005$ (Student's *t* test).

deferens. [$^3$H]-penbutolol accumulation by the tissue was greater than that of NA at each interval. The presence of DMI in a concentration of 1 × $10^{-8}$M did not alter the accumulation curve but pretreatment of rats with 6-OHDA significantly reduced accumulation at 30 min ($P<0.01$).

### *Overflow studies*

Tritium overflow following transmural stimulation of the isolated rat vas deferens was significantly greater than the non-stimulated overflow after incubation with [$^3$H]-NA (sign test, $P<0.001$), see Figure 3. This was not so for [$^{14}$C]-sorbitol (Figure 3), [$^{14}$C]-practolol, [$^{14}$C]-(±)-propranolol or [$^3$H]-penbutolol.

### *Electron microscopy and fluorescence histochemistry*

6-OHDA-induced morphological alterations in the adrenergic nerve endings in the vas deferens of the rat were similar to those previously described by Thoenen & Tranzer (1968) and Malmfors (1971). Electron microscopic studies showed the presence of degenerative changes in the majority of adrenergic nerve endings whereas the surrounding Schwann cell and smooth muscle cells remained intact.

The histofluorescence technique showed a marked decrease in the number and intensity of adrenergic nerve varicosities, findings which were consistent with the electron microscopic studies.

### *The adrenergic neurone blocking effect of penbutolol*

In six experiments penbutolol (1.46 × $10^{-6}$ to 8.76 × $10^{-6}$M) caused a progressive impairment of the

**Table 1** 'Drug space' (µl/mg tissue) of rat vasa deferentia after incubation with [$^{14}$C]-sorbitol, [$^{14}$C]-practolol, [$^{14}$C]-propranolol and [$^3$H]-penbutolol in control vasa, vasa incubated in presence of desmethylimipramine (DMI, 1 × $10^{-8}$ M) and in vasa of rats pretreated with 6-hydroxydopamine (6-OHDA)

| | | *Incubation time (minutes)* | | | |
|---|---|---|---|---|---|
| *Drug* | *Condition* | *5* | *10* | *20* | *30* |
| [$^{14}$C]-sorbitol | Control | 0.347 ± 0.02 | 0.497 ± 0.02 | 0.848 ± 0.08 | 0.690 ± 0.03 |
| | DMI | 0.403 ± 0.02 | 0.604 ± 0.05 | 0.709 ± 0.07 | 0.579 ± 0.12 |
| | 6-OHDA | | | | 0.794 ± 0.09 |
| [$^{14}$C]-practolol | Control | 0.070 ± 0.004 | 0.153 ± 0.010 | 0.281 ± 0.009 | 0.379 ± 0.014 |
| | DMI | 0.059 ± 0.001 | 0.134 ± 0.010 | 0.240 ± 0.022 | 0.329 ± 0.017 |
| | 6-OHDA | | | | 0.357 ± 0.008 |
| [$^{14}$C]-propranolol | Control | 1.79 ± 0.07 | 2.92 ± 0.39 | 4.49 ± 0.51 | 6.31 ± 0.12 |
| | DMI | 2.12 ± 0.14 | 3.51 ± 0.08 | 5.30 ± 0.32 | 5.94 ± 0.60 |
| | 6-OHDA | | | | 5.55 ± 0.54 |
| [$^3$H]-penbutolol | Control | 2.30 ± 0.14 | 5.11 ± 0.17 | 7.05 ± 0.55 | 10.45 ± 0.52 |
| | DMI | 2.57 ± 0.07 | 6.13 ± 0.79 | 7.33 ± 0.35 | 10.47 ± 0.37 |
| | 6-OHDA | | | | 8.06 ± 0.81 |

Figures are means ± s.e. mean of at least four observations

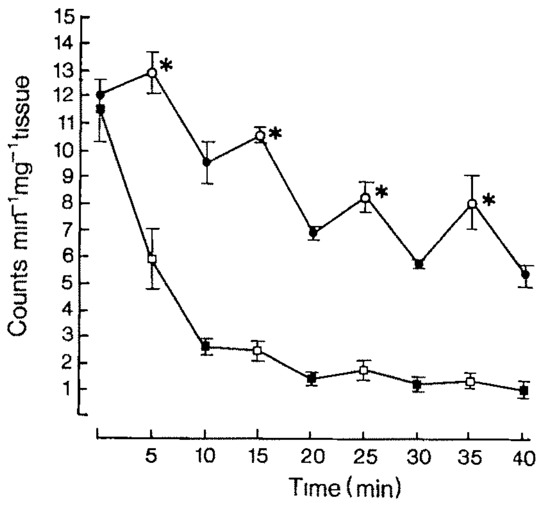

**Figure 3** Overflow pattern of [$^{14}$C]-D-sorbitol (■-□), and [$^3$H]-(−)-noradrenaline (●-○) from the isolated vas deferens of the rat at rest (closed symbols) and following transmural stimulation (open symbols). Each point is the mean of results from 6 vasa. Vertical lines show s.e. mean Vertical axis· counts min$^{-1}$ mg$^{-1}$ tissue; horizontal axis· Sampling times (min). * Significant increase in overflow.

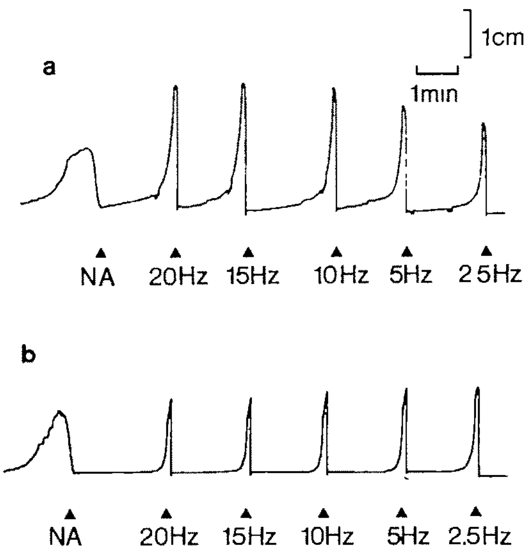

**Figure 4** The effect of noradrenaline (NA, $2.96 \times 10^{-5}$M) and transmural stimulation (2.5 to 20 Hz supramaximal voltage) on the contractions of the isolated vas deferens of the rat. (a) Shows control responses and (b) responses in the presence of penbutolol $8.76 \times 10^{-6}$M

responses to sympathetic nerve stimulation whilst the responses to added NA were either unaffected or occasionally increased. The result of a typical experiment using penbutolol in a concentration of $8.76 \times 10^{-6}$M is shown in Figure 4.

## Discussion

The carbohydrate, D-sorbitol, is known to be distributed exclusively in the extracellular space (Morgan, Henderson, Regan & Park, 1961) and the present results show that this distribution is not altered significantly by the presence of DMI or pretreatment with 6-OHDA. In contrast, NA is actively accumulated by adrenergic neurones by the uptake$_1$ process (Iversen, 1967) and as can be seen from the results, this accumulation is significantly reduced at 30 min by the uptake$_1$-blocking agent, DMI and by pretreatment with 6-OHDA, a drug known to destroy adrenergic neurones (Thoenen & Tranzer, 1968). The accumulation of practolol or propranolol was not affected significantly by DMI or pretreatment of the animals with 6-OHDA. One may conclude, therefore, that neither of these drugs is being taken up into noradrenergic neurones.

The retention of [$^3$H]-penbutolol by the rat isolated vas deferens was greater at each incubation than that of any of the other drugs studied. The accumulation of this β-adrenoceptor blocking agent at the 30 min incubation time (the only time tested) was significantly less after pretreatment of the rats with 6-OHDA. However, its accumulation was not affected by DMI, $1 \times 10^{-6}$M. It would appear, therefore, that penbutolol is partly accumulated by the rat vas deferens by uptake into noradrenergic neurones like NA but its route of entry into neurones may not be via the uptake$_1$ process. The markedly greater affinity of penbutolol for the tissue relative to NA implies either other sites of binding for this drug than those that are available for NA or a greater affinity of penbutolol for the same binding sites.

Stimulation of the sympathetic nerves in the vas did not enhance the overflow of the β-adrenoceptor blocking agents as it did that of NA, and in this respect their overflow patterns resembled that of sorbitol.

Propranolol has previously been shown to possess adrenergic neurone blocking activity (Barrett & Nunn, 1970) and from the present investigation penbutolol has also been shown to exert this activity in the isolated vas deferens of the rat. Practolol, however, has no adrenergic neurone blocking activity in acute studies (Barrett & Nunn, 1970) but in chronic dosage does reduce the blood pressure response of the pithed rat to spinal cord stimulation (Lewis, 1974b). Penbutolol behaves like guanethidine in being taken up by adrenergic neurones but differs from guanethidine in that its uptake into neurones is not blocked by DMI nor is it released from sympathetic neurones following electrical stimulation. However, practolol and propranolol appear not to be taken up into noradrenergic neurones and one must conclude that any adrenergic neurone blocking effects which they possess are exerted from outside the neurone. This effect would seem to be produced by a mechanism which does not involve either their uptake or subsequent release from adrenergic neurones.

In conclusion, it appears that any effect on the adrenergic neurone brought about by practolol and (±)-propranolol occurs without these drugs gaining access to the interior of the neurone. It may be that the mechanism of the adrenergic neurone blocking action of penbutolol is different, as it is accumulated in the neurone, whereas the other $\beta$-adrenoceptor blocking agents are not.

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# THE EFFECTS OF ETHER STRESS AND BETAMETHASONE TREATMENT ON THE CONCENTRATIONS OF NORADRENALINE AND DOPAMINE IN VARIOUS REGIONS OF THE RAT BRAIN

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1 The effects of ether stress on noradrenaline (NA) and dopamine levels in different regions of the rat brain were studied.

2 Exposure to ether vapour (90 s) caused a significant decrease in the concentration of hypothalamic NA but had no effect on catecholamine (CA) concentrations in the other regions studied.

3 Treatment with betamethasone alone (20 µg/ml) given in the drinking water for 24 h, had no effect on CA levels in the cerebral cortex, midbrain or hypothalamus. However, pretreatment with this dose of steroid prevented the decreases in hypothalamic NA which were normally seen after ether stress and also induced significant increases in the concentration of midbrain NA.

4 The data provide further support for the involvement of NA in the regulation of stress-induced corticotrophin (ACTH) release and indicate that centres other than the hypothalamus may be involved in mediating the inhibitory action of betamethasone on the response to ether stress.

## Introduction

Central monoamines have been implicated in the regulation of corticotrophin (ACTH) release, and are found in fairly high concentrations in those regions of the brain that are believed to play a role in this process (e.g. the hypothalamus and midbrain).

Various forms of stressful stimuli have been shown to affect brain catecholamine (CA) levels, although the literature contains some conflicting views. In general, noradrenaline (NA) concentrations have been found to decrease (Ganong & Lorenzen, 1967; Bliss, Ailion & Zwanziger, 1968; Carr & Moore, 1968; Stoner & Elson, 1971), whereas concentrations of dopamine have been reported to be either unchanged (Carr & Moore, 1968; Corrodi, Fuxe, Lidbrink & Olson, 1971) or decreased (Gordon, Spector, Sjoerdsma & Udenfriend, 1966) after stress.

However, most studies of the effects of stress on brain CA concentrations have employed techniques that involve modification of endogenous brain CA levels by the administration of chemical substances, followed by the assessment of pituitary-corticotrophic function before and after stress (Van Loon, Scapagnini, Moberg & Ganong, 1971; Scapagnini, Van Loon, Moberg, Preziosi & Ganong, 1972) or have looked only at CA concentrations in whole brain or limited regions (Barchas & Freedman, 1963). The present study involved the direct estimation of NA and dopamine in seven different regions of the rat brain. It was carried out in order to investigate the effects of a known stressful stimulus (exposure to ether vapour) and the effects of a known inhibitor of the ACTH-releasing activity of this stress (betamethasone), on the concentrations of these neurotransmitter substances.

## Methods

### *Animals*

Adult male albino Charles River rats, weighing 200–250 g were used throughout. The animals were housed 2 per cage, at least 5 days before the start of an experiment, and were handled 3 times per week from then onwards (Hodges & Mitchley, 1970b). Food and water were available *ad libitum*.

### *Stress*

The animals were stressed at 09 h 15 min, by placing them in a closed box into which ether vapour was passed. Each animal was exposed to the vapour for

**Table 1** Reproducibility of dissection procedure

| *Brain region* | *Mean wt. (mg ± s.e. mean)* |
|---|---|
| Cerebral cortex | 212 ± 6 |
| Cerebellum | 282 ± 5 |
| Pons and medulla | 218 ± 6 |
| Hypothalamus | 51 ± 2 |
| Midbrain | 131 ± 4 |
| Corpus striatum | 77 ± 2 |
| Hippocampus | 96 ± 2 |

Mean weights for various brain regions dissected from 36 control animals.

90 s, this being sufficient to induce anaesthesia. At specific times thereafter the animals were killed by decapitation. The brains were rapidly removed, chilled in methanol which had been pre-cooled to −60°C by an acetone/solid $CO_2$ mixture, and kept on solid $CO_2$ until all the animals in a given experimental group had been killed. A group of 6 untreated control animals was also killed at each time.

### *Brain dissection and catecholamine assay*

The following brain regions were dissected out, according to the scheme outlined by Glowinski & Iversen (1966): cerebral cortex, cerebellum, pons and medulla, midbrain, hypothalamus, corpus striatum and hippocampus. The tissue samples were weighed accurately on a torsion balance and homogenized in 2.5 ml of ice-cold 0.4 N perchloric acid. The reproducibility of the dissection procedure is shown in Table 1. NA and dopamine concentrations were determined fluorimetrically according to the detailed method described by Shellenberger & Gordon (1971).

### *Betamethasone*

Betamethasone in a soluble form (betamethasone sodium phosphate, Glaxo Laboratories Ltd) was administered to rats in the drinking water at a concentration of 20 μg/ml, for a period of 24 hours. Preliminary experiments showed that the total volume of drug solution ingested over the 24 h period remained constant and was equivalent to 450–500 μg of drug/100 g body weight (Vellucci, unpublished observations). The administration of betamethasone in the drinking water provides a non-stressful method of administering the drug and obviates the need for injections. This method was first described by Purves & Sirett (1965) for dexamethasone, and has since been used by other workers in this field (Hodges & Mitchley, 1970a, c; Buckingham & Hodges, 1976).

## Results

### *The effects of ether stress on the concentrations of noradrenaline and dopamine*

Of the seven brain regions studied, it was found that significant changes in concentration occurred only in the hypothalamus (Table 2). In this region the concentration of NA fell significantly ($P < 0.01$) from a control value of 6.1 nmol/g, to reach a value of 3.8 nmol/g 10 min after exposure to the stress. The NA concentration then rose to 5.4 and 5.3 nmol/g after 20 and 40 min respectively, but remained significantly ($P < 0.05$) below the control level. It then fell again to reach a value of 3.6 nmol/g at 160 min, which was significantly ($P < 0.001$) lower than that of the corresponding unstressed controls.

Dopamine concentrations were also estimated in the same samples. In all cases the dopamine concentrations were found to remain at a level which did

**Table 2** The effect of ether stress on the concentration of noradrenaline (nmol/g tissue wet weight) in different regions of the rat brain

| | *Time (min) after exposure to ether* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *0* | *10* | *20* | *40* | *80* | *160* | *320* |
| *Brain region* | (30) | (12) | (6) | (12) | (12) | (12) | (12) |
| Cerebral cortex | 1.2 ± 0.07 | 1.2 ± 0.1 | 1.0 ± 0.1 | 1.2 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 | 0.9 ± 0.1 |
| Cerebellum | 0.8 ± 0.08 | 0.7 ± 0.1 | 1.0 ± 0.1 | 0.9 ± 0.06 | 0.8 ± 0.04 | 0.8 ± 0.07 | 0.9 ± 0.1 |
| Pons + medulla | 1.5 ± 0.07 | 1.5 ± 0.1 | 1.6 ± 0.3 | 1.3 ± 0.1 | 1.7 ± 0.2 | 1.2 ± 0.1 | 1.4 ± 0.1 |
| Hypothalamus | 6.1 ± 0.1 | ***3.8 ± 0.4 | *5.4 ± 0.2 | *5.3 ± 0.3 | **4.9 ± 0.1 | ***3.6 ± 0.1 | ***4.7 ± 0.1 |
| Midbrain | 2.3 ± 0.2 | 1.9 ± 0.3 | 2.5 ± 0.3 | 2.4 ± 0.3 | 2.1 ± 0.1 | 2.1 ± 0.2 | 2.0 ± 0.3 |
| Corpus striatum | 1.2 ± 0.2 | 1.3 ± 0.3 | 1.3 ± 0.1 | 1.4 ± 0.05 | 1.5 ± 0.2 | 1.3 ± 0.1 | 1.4 ± 0.2 |
| Hippocampus | 1.8 ± 0.2 | 1.7 ± 0.2 | 1.5 ± 0.3 | 1.7 ± 0.2 | 1.8 ± 0.2 | 1.5 ± 0.2 | 1.8 ± 0.2 |

Numbers of animals indicated in parentheses. Values are means ± s.e. mean

Significantly different from controls, *$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

**Table 3** Dopamine concentrations (nmol/g tissue) in different regions of the rat brain

| *Brain region* | *Dopamine* |
|---|---|
| Cortex | 0.8±0.1 |
| Cerebellum | —* |
| Pons+medulla | 2.3±0.3 |
| Hypothalamus | 2.4±0.3 |
| Midbrain | 0.4±0.05 |
| Corpus striatum | 22.0±3.6 |
| Hippocampus | —* |

Each value is the mean (±s.e. mean) of 6 control groups, each containing 6 animals.

* For the cerebellum and hippocampus the fluorimeter readings were not significantly different from the blank value.

not differ significantly from that of the corresponding control groups (Table 3).

The hypothalamic weights of the stressed animals were not significantly different from those of the corresponding controls.

### *The effect of betamethasone treatment on the response to ether stress*

Buckingham & Hodges (1976) have shown, by the use of direct estimates of circulating ACTH, that pretreatment with betamethasone inhibits the stress-induced release of this trophic hormone. Thus, it was of interest to investigate the effects of pretreatment with a suitable dose of betamethasone on the CA response to ether stress. Three brain areas were selected for this study, viz. the cerebral cortex, the midbrain and the hypothalamus, although this does not imply that the other regions are unimportant.

In the cerebral cortex no change occurred in the concentration of NA after betamethasone alone or after betamethasone and ether stress (Figure 1a).

In the midbrain (Figure 1b) neither treatment with betamethasone alone, nor ether stress alone had any effect on the concentration of NA. However, in the groups pretreated with the steroid and then exposed to ether stress there was a significant increase in the concentration of NA, from a control value of 2.3 nmol/g to values of 2.9 nmol/g ($P<0.01$) and 3.2 nmol/g ($P<0.001$) after 10 and 160 min, respectively.

Betamethasone alone had no significant effect on hypothalamic NA (Figure 1c). However, the fall in hypothalamic NA which was normally seen 10 and 160 min after exposure to ether vapour was completely inhibited by pretreatment with the steroid. Furthermore, there was a slight, but significant ($P<0.05$) increase in hypothalamic NA in the steroid-treated group 10 min after stress.

Dopamine levels were also estimated in the same samples. No changes in the concentration of the amine were observed and all values remained at levels which were not significantly different from those of the corresponding controls.

The hypothalamic weights did not differ significantly between groups.

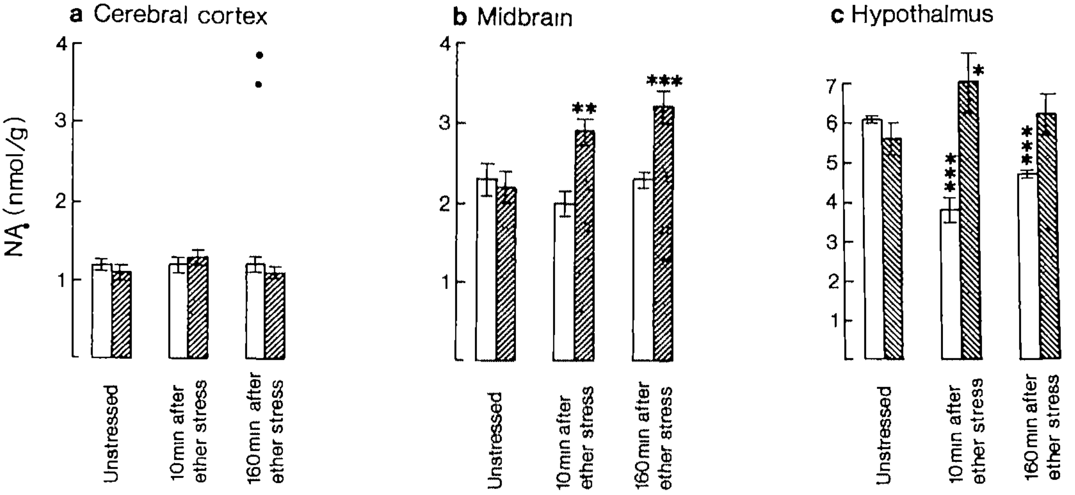

**Figure 1** Brain noradrenaline concentrations in untreated rats (open columns) and betamethasone-treated rats (hatched columns) before and after ether stress in (a) cerebral cortex, (b) midbrain and (c) hypothalamus. Each column is the mean of at least 8 determinations. Vertical bars indicate s.e. mean.

Significantly different from corresponding controls. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

## Discussion

These results show that a significant decrease in the concentration of hypothalamic NA occurred after ether stress, whereas NA levels in the other brain regions, and dopamine levels in all regions, remained unchanged. In addition they show that pretreatment with betamethasone was able to prevent this stress-induced decrease in hypothalamic NA. The results are in agreement with the findings of several authors who have found a decrease in hypothalamic NA after a variety of other stressful stimuli, e.g. emotional stress, foot-shock, forced swimming, restraint, and injection of histamine (Bliss & Zwanziger, 1966; Bliss *et al.*, 1968; Stoner & Elson, 1971), and with those of authors who have found no change in the concentration of dopamine after stress (Bliss *et al.*, 1968; Carr & Moore, 1968; Corrodi *et al.*, 1971). They are also in agreement with the observation that pretreatment of rats with hydrocortisone (cortisol) is capable of preventing the fall in brain NA content which is normally induced by the stress of an electric shock applied to the feet (Shalyapina, 1967).

The results described here support the theory that NA is involved in the regulation of stress-induced ACTH release. However, they do not indicate precisely how NA is involved in the regulation of stress-induced ACTH release. Further studies are therefore necessary in order to determine the relative contribution made by changes in synthesis and/or release of NA and to correlate these changes with alterations in hypothalamo-pituitary adrenal activity. In the light of the recent findings of Palkovits, Kobayashi, Kizer, Jacobowitz & Kopin (1975) who demonstrated that the CA content of the arcuate nucleus was selectively depleted after exposure to three different types of acute stress (i.e. formalin, immobilization and exposure to cold), it would also be of interest to determine whether the decrease in hypothalamic NA observed after ether stress is localized in this hypothalamic nucleus. In the present work only hypothalamic NA exhibited a significant decrease after ether stress, however, it has been reported that a more severe stress, such as foot-shock, can induce decreases in NA throughout the entire brain (Bliss *et al.*, 1968). In addition it cannot be concluded from the present results that CA function in the other regions of the brain remained unaltered, as it is possible for CA turnover rates to change quite markedly without concomitant changes in their absolute levels (Bliss *et al.*, 1968).

It is well established that CA concentrations in different regions of the brain exhibit some degree of diurnal variation (Manshardt & Wurtman, 1968; Simon & George, 1975). However, the observed decreases in hypothalamic NA were thought not to be due to this. This is because, firstly, concentrations of hypothalamic NA have been shown to remain relatively stable during the period of time when the experiments were carried out (Simon & George, 1975), and secondly, because control measurements were made each time and were found not to differ significantly from each other. It is also possible that the observed decreases in hypothalamic NA could have been due to an increase in the blood-content of the hypothalamus. However, no significant differences between the hypothalamic weights of experimental and control animals were found.

Another possible explanation for the finding that NA changes were localized to the hypothalamus, is that ether stress is thought to stimulate ACTH release by acting directly on this region, without the mediation of other parts of the central nervous system (CNS) (Matsuda, Kendall, Duyck & Greer, 1963; Matsuda, Duyck, Kendall & Greer, 1964; Kendall, Allen & Greer, 1965). This seems unlikely, particularly as other investigators have provided evidence which indicates that the response to ether stress is not mediated solely by the hypothalamus, but involves regions such as the midbrain (Royce & Sayers, 1958; Mangili, Motta, Muciacca & Martini, 1965). Mangili *et al.*(1965) found that the corticotrophic response to a stress such as ether was blocked by midbrain transection or by treatment with dexamethasone. Furthermore, Kendall *et al.* (1965) found that, although an increase in plasma corticosterone concentration still occurred in midbrain transected rats subjected to ether stress, it was significantly less than that seen in intact controls, thus indicating that other regions of the CNS are probably necessary for a normal response to this stress. The results reported here demonstrate that whereas neither ether stress, nor betamethasone alone had any effect on midbrain NA levels, betamethasone treatment followed by ether stress induced a significant increase in the NA concentration of this region. Although these results do not show conclusively that betamethasone and/or ether stress exert their effect directly on the midbrain they tend to indicate that this region may play a role in mediating the inhibitory effects of this synthetic steroid on the response to ether stress. This idea is supported by the finding that steroid-sensitive neurones are present in the midbrain, as well as in the hypothalamus (Steiner, Ruf & Akert, 1969).

These findings also emphasize the need for adequate controls when investigations of changes in brain NA function are carried out during, or shortly after, ether anaesthesia.

In summary, the results described here tend to support the theory of NA involvement in the regulation of stress-induced ACTH release. Furthermore, they indicate that the midbrain as well as the hypothalamus may be involved in mediating the inhibitory effects of betamethasone on the response to ether stress. Although direct estimates of CA levels are adequate for a preliminary investigation, more detailed and more localized studies of CA metabolism are also necessary.

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# THE EFFECT OF ZINC ON ANAPHYLAXIS *in vivo* IN THE GUINEA-PIG

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The protective effects of pretreatment with zinc sulphate aerosols against bronchoconstriction induced by egg albumen or histamine aerosols were assessed in sensitized or non-sensitized guinea-pigs respectively. Pretreatment with an adequate concentration of zinc sulphate aerosol significantly prolonged the time of onset of bronchoconstriction in sensitized guinea-pigs challenged with egg albumen, but did not appreciably alter the onset time of histamine-induced bronchoconstriction in non-sensitized animals. These findings suggest that zinc aerosols may be of prophylactic value against bronchoconstriction of allergic origin.

**Introduction** It is generally believed that anaphylactic reactions are produced by the release of autacoids from target cells, especially mast cells, as a consequence of antigen-antibody interaction (Austen, 1974). Inhibition of histamine and slow reacting substance of anaphylaxis (SRS-A) release from the mast cells, e.g. by disodium cromoglycate, has been shown to be effective in the prophylactic treatment of bronchial asthma (Dykes, 1974; Garland & Mongar, 1974).

*In vitro* experimental evidence indicates that zinc ions selectively stabilize the membrane of mast cells from the peritoneum or lungs of rats, and significantly inhibit disruption of these cells by compound 48/80, lecithinase A or antigen-antibody interactions to prevent histamine release (Högberg & Uvnäs, 1960; Kazimierczak & Maśliński, 1974). Thus, it is possible that zinc ions might have a cromoglycate-like action against bronchoconstriction following anaphylaxis.

The purpose of this investigation was to study the effects of pretreatment with zinc sulphate aerosols on bronchoconstriction induced by anaphylaxis *in vivo* in guinea-pigs.

**Methods** Guinea-pigs of either sex, weighing 400–550 g, were used. One group was sensitized to egg albumen by an intramuscular injection of 0.7 ml of a 5% solution of crystalline egg albumen (BDH), prepared in 0.9% w/v NaCl (saline), 3 weeks before challenge (Herxheimer, 1952). At the start of the experiment, each animal was put into a plastic observation chamber (350 × 190 × 280 mm) and anaphylaxis was induced by an aerosol of the same solution of antigen introduced into the closed chamber. The onset of marked dyspnoea was taken as the end-point, and the duration of exposure required to produce bronchoconstriction was recorded. In the group of non-sensitized guinea-pigs, bronchoconstriction was induced by an aerosol of freshly prepared 2% w/v histamine diphosphate (expressed as the salt; Sigma) in saline (final pH 4.1). A finely atomized mist of either egg albumen or histamine solution was produced by a nebulizer connected to a compressor pump (pressure 120 mmHg). The solutions were atomized at a rate of 0.025 ml/minute.

The protective effect of zinc sulphate ($ZnSO_4.7H_2O$; May & Baker) against bronchoconstriction induced by aerosols of egg albumen in sensitized guinea-pigs or of histamine in non-sensitized animals was assessed by exposing the animals to an aerosol of this agent for 5 min immediately before starting the experiments. Zinc sulphate was dissolved in saline (20, 40 or 80 mg/ml w/v; expressed as the salt including its water of crystallization) before use (final pH 4.8). Th pH of saline aerosols given to the controls was adjusted to 4.8 to obviate possible bronchial tissue pH changes which might interfere with the release, diffusion or activity of histamine following anaphylaxis, or its diffusion and activity following inhalation.

**Results** In sensitized guinea-pigs, challenge with an aerosol of 5% egg albumen produced anaphylaxis characterized by marked dyspnoea. Pretreatment with zinc aerosols prolonged the time of onset of dyspnoea, and statistical significance was reached with concentrations of 40 and 80 mg/ml ($P < 0.01$ for both, Table 1).

In non-sensitized guinea-pigs, exposure to an aerosol of 2% histamine also induced bronchoconstriction characterized by marked dyspnoea. However, pretreatment with zinc aerosols, at concentrations which significantly protected sensitized animals from anaphylaxis, did not appreciably alter the time of onset of dyspnoea.

**Discussion** The ability of pretreatment with zinc aerosols to delay significantly the onset of bronchoconstriction induced by antigen challenge in sensitized guinea-pigs is probably due to membrane stabilization

**Table 1** The effects of pretreatment with zinc aerosols against bronchoconstriction induced by aerosols of egg albumen in sensitized guinea-pigs or of histamine in non-sensitized guinea-pigs

| | *Exposure time required to produce bronchoconstriction (min)* | |
|---|---|---|
| *Pretreatment* | *Egg albumen (5%)* | *Histamine diphosphate (2%)* |
| Saline | 2.81 ± 0.23 (8) | 1.39 ± 0.09 (12) |
| Zinc sulphate | | |
| 20 mg/ml | 3.26 ± 0.14 (8) | – |
| 40 mg/ml | 4.83 ± 0.53 (8)* | 1.45 ± 0.12 (11) |
| 80 mg/ml | 4.25 ± 0.37 (8)* | 1.52 ± 0.09 (12) |

Each value is the mean ± s.e mean of the number of animals given in parentheses. *$P<0.01$ when compared with the saline-pretreated control using Student's *t* test.

of the mast cells, and consequent inhibition of the release of autacoids by the antigen-antibody interaction (Mota & Vugman 1956; Högberg & Uvnäs, 1960). This effect appears to be similar to that produced by cromoglycate when used for bronchial asthma in man. However, pretreatment with cromoglycate aerosols, with concentrations as high as 100 mg/ml, did not significantly delay the onset of antigen-induced bronchoconstriction (unpublished observations); this is in accord with the observation of Assem & Mongar (1970) that cromoglycate is unable to prevent anaphylactic release of histamine from guinea-pig lungs. Our findings are not at variance with the idea that zinc may have acted through a different mechanism to stabilize the mast cell membrane.

The possibility of zinc acting as a physiological antagonist, or as a local irritant by stimulating mucus secretion in the bronchial tree to prevent contact of the antigen with the bronchial mucosa, cannot be excluded. These effects are unlikely because pretreatment with zinc aerosols did not protect the non-sensitized guinea-pigs from bronchoconstriction induced by histamine aerosols.

Our results suggest that zinc aerosols may be useful for preventing bronchoconstriction in anaphylactic reactions and in bronchial asthma. However, since cromoglycate is ineffective in guinea-pigs, the possibility of species difference in the activity of zinc has also to be borne in mind.

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Papers will be considered for publication on all aspects of drug action, including chemotherapy. A recent issue of the Journal is a good guide to style.

Manuscripts (two copies) should be sent to Dr A. Ungar, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh, EH8 9JZ, from whom copies of the full Instructions to Authors, published in *Br. J. Pharmac,* 50, 3–23, may also be obtained. Specific points to note are:

1. **Manuscript** should be typed in double spacing on one side of paper not larger than A4 (206 x 294 mm). There should be a separate title page giving the names and addresses of the authors in alphabetical order. A short title of not more than 50 letters should also be suggested.

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